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Archaea at the El Tatio Geyser Field: community composition, diversity, and distribution across hydrothermal features and geochemical gradients

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Archaea at the El Tatio Geyser Field: community composition, diversity, and distribution across hydrothermal features and geochemical gradients

by

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Archaea at the El Tatio Geyser Field: community composition, diversity, and distribution across hydrothermal features and geochemical gradients

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Methanogenesis, a metabolic pathway unique to Archaea, is severely inhibited by the reduced form of arsenic (As). Despite this inhibition, methanogenic Archaea are present in some hydrothermal features at the El Tatio Geyser Field (ETGF), a high-arsenic site with 100+ hydrothermal features, including boiling pools, geyers, fumaroles, and springs. The ability of methanogenic Archaea and other microorganisms to withstand elevated arsenic concentrations, and a variety of other extreme environmental conditions at ETGF, may be due to unique adaptations or syntrophic relationships with other microorganisms.

ETGF is situated in the Andes Mountains at an altitude of ~4300 meters. UV radiation is elevated in this region and air temperatures fluctuate widely. Most hydrothermal waters discharge at ~85˚C, the local boiling point, and rapidly evaporate due to the arid climate. This concentrates hydrothermal salts and metals, including arsenic (As) and antimony (Sb). Additionally, dissolved inorganic carbon (DIC) concentrations are extremely low in most features and may limit life.

Water chemistry analyses done for this study show variability in dissolved constituents between features that are consistent over time. Variations may be due to the source or residence time of waters, and differences in chemistry could be responsible for the presence or absence of methanogenic Archaea at hydrothermal sites. The overlying control on microbial diversity and community composition may be water geochemistry, and potentially specific constituents.

The goals of this study were to detect novel microbial taxa at ETGF, including novel methanogens, as well as to document microbial community composition at select hydrothermal features. The distribution and diversity of microorganisms at each feature was analyzed phylogenetically and within an ecological context in order to determine physicochemical and biological controls on community composition. Additionally, a model methanogen was used in laboratory analyses to determine how concentrations and oxidation states affected growth and methane production. This methanogen, *Methanothermobacter thermautotrophicus,* is found at ETGF, Yellowstone, and other hydrothermal fields, and thrives in high-temperature environments.

MPN (most probable number) analyses show that culturable biomass from multiple sites contain metabolically active methanogens. These results support the biogenicity of dissolved methane detected in the field. 16S rRNA surveys of Archaea at four sites show that Archaea are diverse, and archaeal community composition varies across features. Phylogenetic tree construction indicates that Archaea from ETGF group together, suggesting that the isolation and broad environmental constrains on ETGF have some control on phylogenetic diversity.

Laboratory analyses of As and Sb concentrations on *M. thermautotrophicus* suggest that Sb may decrease the inhibition of methanogenesis by As by preventing the formation of As(III) from As(V). Statistical analyses correlating microbial community composition and structure to physicochemical parameters show that archaeal and bacterial communities relate to different variables; with Bacteria correlating to water temperature, and Archaea correlating to dissolved constituents such as hydrogen gas and sulfate.

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Chapter 1: Introduction

HYDROTHERMAL SYSTEMS AS ANALOGUES FOR EARLY LIFE SYSTEMS

Hydrothermal systems: The first microbial habitats?

Geologic and microfossil evidence consistently show that early life was initially dominated by thermophilic prokaryotes. Hydrothermal activity was abundant on the early Earth, and thermophilic microorganisms may have lived within either terrestrial or deep-sea hydrothermal systems (Kletzin, 2007; Konhauser et al., 2003; Madigan & Martinko, 2006; Orange et al., 2009). Phylogenetic data also support this hypothesis, because deeply rooted Archaea and Bacteria are thermophilic and have anaerobic chemoheterotrophic or chemolithoautotrophic metabolisms (Amend & Shock, 2001; Konhauser, 2007; Konhauser et al., 2003; Woese et al., 1990), which could have been sustained by hydrothermal energy sources.

Evidence from the rock record suggests that microbial mat communities lived in shallow water systems prior to the formation of ozone (Allwood et al., 2006), despite damaging levels of UV radiation. Water is an insufficient attenuator of UV; 30 meters of ocean water would have been required to reduce UV radiation to levels experienced on the surface today (Phoenix et al., 2006).

Microbial communities must have had some protection from DNA-damaging UV radiation prior to the formation of oxygen-derived ozone in the atmosphere. Early microbial ecosystems may have been tied to hydrothermal systems not only as a sources of electron donors for chemotrophy (Des Marais, 2000), but also as protection from UV

radiation. The formation of microbial mats, as well as the precipitation of hydrothermal minerals, both may have contributed to the capacity of hydrothermal microbial communities to survive high UV radiation.

In terrestrial hydrothermal systems, silica, iron, and other minerals can precipitate from supersaturated waters and nucleate on cyanobacterial sheaths and other microbial structures. Precipitating silica and ferric iron create a protective coating that absorbs UV radiation yet still allows PAR to penetrate and photosynthesis to occur (Phoenix et al., 2001). Other mechanisms for protecting cells from UV radiation, such as the formation of microbial mat communities in which dead cells in upper mat layers protect microbes in lower mat layers from UV radiation also may have played a role (Westall et al., 2006).

Mat communities: the first microbial ecosystems

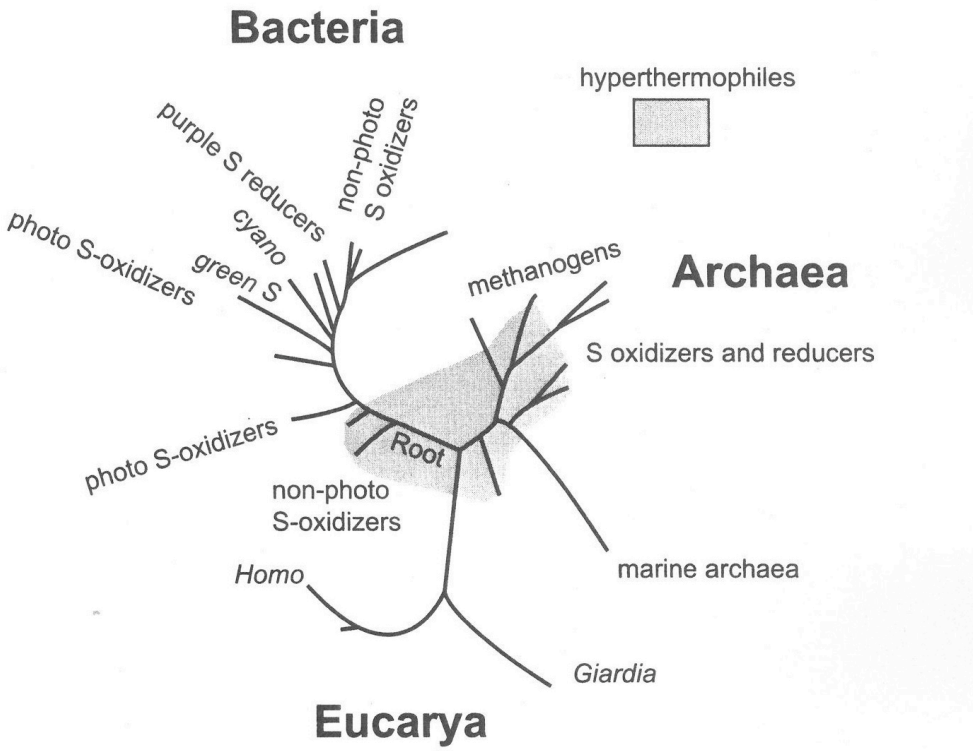
Modern microbial mat communities in extreme environments such as hydrothermal systems are considered self-sustaining, relatively isolated prokaryotic ecosystems (Ward et al., 1998) similar to communities that existed during the anoxic-oxic atmospheric transition on Earth around 2.7 billion years ago (GYA) (Bebout et al., 2004; Paerl et al., 2000). These mat communities are perfect analogs for those seen on the early Earth, because extreme conditions both replicate Archean habitats and minimize eukaryotic interactions. The prokaryotes found in modern mat communities often include deeply rooted taxa, which may be phylogenetically similar to the last universal common ancestor (LUCA)- the proposed organism from which all life evolved. Figure 1.1 is a

16S rRNA phylogenetic tree showing the deeply rooted placement of modern-day hyperthermophilic and thermophilic prokaryotes.

Studies of modern microbial mat communities show metabolically and phylogenetically diverse prokaryotes live in close proximity, often facilitating changes in geochemical conditions over millimeter or smaller scales (Paerl et al., 2000). Fossilized stromatolites from the Archean consist of morphologically distinct prokaryotes (Awramik & Semikhatov, 1979; Orange et al., 2009; Schopf et al., 2007), suggesting that this diversity may have existed in early mat communities as well.

Our ability to determine the phylogenetic composition and metabolic capabilities

Figure 1.1: Position of hyperthermophiles in the 'standard view' of the evolutionary tree based on rRNA (Nisbet, 2002).



of microorganisms in early microbial mat communities is limited, because neither phylogenetic nor direct metabolic information about ancient mat communities can be inferred with fossil evidence. Isotopic evidence from fossilized stromatolites can be used to infer the presence of metabolic by-products and thus give insight into populations inhabiting Archean mat communities (House et al., 2000). Microbially mediated geologic structures, such as banded iron formations, may also give insight into the metabolic capabilities of early life. For example, isotopic evidence indicates Archaea played a dominant role in carbon cycling up to 3.5 GYA (Ventura et al., 2007), and that methanogenic Archaea may have produced sufficient amounts of methane to regulate the Earth's climate during the Archean era (Ueno et al., 2006).

Modern day mat communities typically include oxygenic phototrophs such as cyanobacteria. These microbes are thought to be responsible for the original oxygenation of the atmosphere, an event which initially stressed, but later greatly increased the diversity and productivity of life (Des Marais, 2000; Hoehler et al., 2001). Despite higher oxygen levels in the atmosphere, prokaryotes with anaerobic metabolisms still thrive within mat communities, in which microbial consumption of O_2 creates anaerobic microenvironments. Typically within mat communities, the microenvironment becomes more important than large-scale environmental factors, because microbial metabolisms dictate geochemical conditions.

Because phylogenetic data cannot be directly taken from fossilized microbial communities, sequencing genes from modern analogue sites can indirectly assess the composition of early microbial communities. Although representatives from the domains

Bacteria and Archaea are both incorporated into my study, my focus is Archaea, primarily methanogenic Archaea. Geochemical parameters incorporated within this study include concentrations of arsenic and antimony, as well as dissolved inorganic carbon, temperature, and salinity.

GENETICS, EVOLUTIONARY RELATIONSHIPS, AND MICROBIAL DIVERSITY

How modern genetics changed microbial diversity studies

The advent of nucleic acid sequencing allowed Life to be characterized and organized in ways that were previously impossible: phenotypic characterization gave way to genomic characterization, and determining phylogenetic relationships between unicellular organisms, particularly prokaryotes, became possible for the first time. This new technology was used to divide all life on Earth into three domains, Archaea, Bacteria, and Eucarya (Woese, 1990). Nucleic acid sequencing also opened up the field of prokaryotic evolutionary biology, a field previously ignored due to the difficulties in determining evolutionary relationships between prokaryotes, a group for which phenotypic differences often failed to align with evolutionary distance (Woese, 1987). Widespread lateral gene transfer complicates evolutionary relationships between prokaryotes; however, researchers can utilize multiple genes and/or ribosomal proteins to further test uncertain phylogenetic relationships (Matte-Tailliez et al., 2002; Pace, 1997).

The ability to identify prokaryotes based on their ribosomal RNA (rRNA) alone removed the necessity of laboratory cultivation and allowed for direct classification of prokaryotes in environmental samples (Olsen et al., 1986). Phylogenetic studies of

microorganisms often use rRNA, or genes coding for rRNA (rDNA), as a method of determining evolutionary relationships between microorganisms. A combination of highly conserved and variable regions, along with universal functional and evolutionary homology between rRNAs, makes the small subunit rRNA (16S in prokaryotes and 18S in eukaryotes) an ideal tool for classifying phylogenetic relationships across domains. Additionally, rRNA permits statistically significant comparisons, and minimizes lateral gene transfer between organisms that could confuse evolutionary relationships (Olsen et al., 1986).

Partial 16S rRNA sequence analysis, used to classify prokaryotes, has been shown to approximate whole-genome similarity between organisms (Madigan & Martinko, 2006). Microbial phylotypes are determined by grouping environmental sequences based on their percent similarity. Most phylogenetic databases use 16S rRNA in studies initially establishing community composition, using a cut-off of 97-98% similarity to define a phylogenetic species two organisms, and a cut-off of 95% to assign two organisms to the same genus (Madigan & Martinko, 2006). These phylotypes, called operational taxonomic units (OTUs), substitute for a species (or other taxonomically defined group) in diversity studies (Hughes et al., 2001).

The tree of life and the earliest organisms

When microbial systematics was reorganized to recognize three primary lines of descent (Woese, 1987; Woese et al., 1990), corroborating data had already accumulated. Prior to Woese's 'discovery' of Archaea, major differences between archaeal taxa (then

called archaebacteria) and their supposed closest relatives had been documented. For example, *Halobacterium* was noted to possess unique membrane lipids and a cell wall structure unlike those found in Bacteria (Jones et al., 1987). *Thermoplasma*, another archaeon, was distinguished from Bacteria by histone-like proteins typically found only in Eucarya (Jones et al., 1987). These anomalies blurred the boundaries between Eucarya and Bacteria, and could not be adequately explained until rRNA-based classification arose.

The universal tree shown in Figure 1.2 is rooted based on paralogous gene sequences that diverged prior to the emergence of the three domains (Jurgens, 2002). Although most scientists agree that a prokaryotic domain, either Bacteria or Archaea, emerged first, molecular dating has thus far proved insufficient to determine which lineage is more ancient (Gribaldo & Brochier-Armanet, 2006). Deeply rooted taxa from the domains Archaea and Bacteria are thermophilic or hyperthermophilic, suggesting the LUCA of all life today (the root of the tree) was a thermophilic prokaryote. Whole-genome analysis of *Methanococcus jannaschi* indicated the LUCA had the capacity for autotrophy, because autotrophic pathways shared between Bacteria and Archaea appear to have a common evolutionary origin (Bult et al., 1996). Although Bacteria and Archaea both share traits derived from the LUCA, Archaea and Eucarya later shared a common evolutionary path independent of Bacteria (Figure 1.2).

The most easily recognizable difference in Archaea and Bacteria is in the structure of the lipid bilayer in the cell membrane. Archaeal membranes are composed of phytanyl ether lipids rather than the ester-linked fatty acids found in bacterial

membranes. This difference, along with proteinaceous S layer found in most Archaea, may account for the increased presence of Archaea in extreme environments. Inside the cell, archaeal chromosomes are circular and resemble those of Bacteria. However, the proteins responsible for DNA replication are more similar to those found in Eucarya (Kletzin, 2007). Transcription in Archaea resembles that of Eucarya, although the regulatory proteins are similar to Bacteria, and translation processes are similar to Bacteria but with Archaea-specific features including anitobiotic resistence (Kletzin, 2007). In other words, Archaea shares traits with both Eucarya and Bacteria, and it is difficult to tell with current data which traits are ancestral and which are derived (Gribaldo & Brochier-Armanet, 2006).

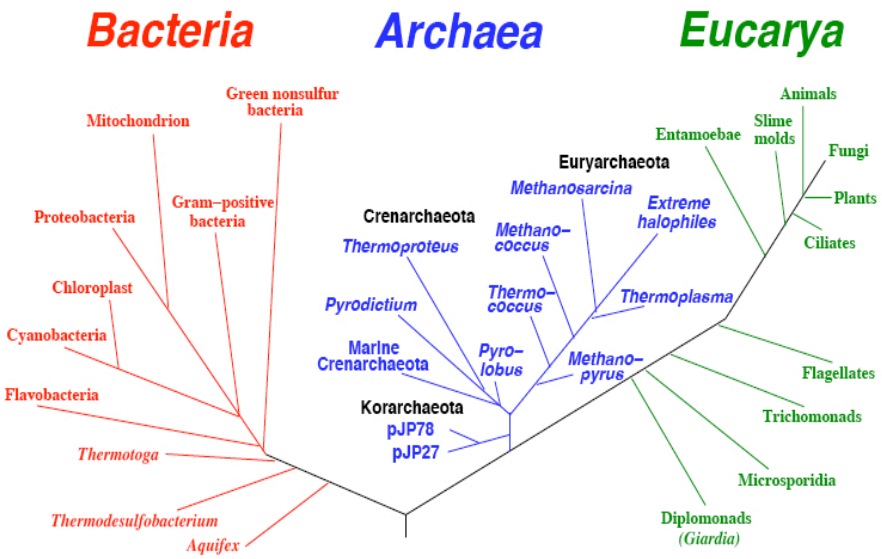


Figure 1.2: Rooted 16S rRNA phylogenetic tree showing the three domains of life. Root represents the last universal common ancestor (LUCA) (Jurgens, 2002).

GEOMICROBIOLOGY AT HYDROTHERMAL SYSTEMS

Microbial Ecology

Microbial ecology deals with the interrelationships between microorganisms, as well as between microorganisms and their environment. Geomicrobiology puts microbial ecology into a geologic context, and incorporates the relationships that influence geochemical reactions, the cycling of organic and inorganic matter, and the formation and degradation of rocks and minerals (Ehrlich & Newman, 2009).

For geomicrobiologists, the metabolic capabilities of microorganisms often are more important than phylogenetic identification, because metabolic by-products are responsible for altering water chemistry and mineral dissolution/precipitation. Assigning a specific metabolic function to a phylogenetic group of microorganisms is problematic; therefore, although the metabolic function of a community may be easy to ascertain, determining which microorganisms within the community are performing that function is more difficult. The genetic relationship between organisms with similar metabolic processes may show evolutionary relationships, such as the orthologous or paralogous nature of certain genes, which could contribute to understanding the effects of biogeography, competition, and other factors on microbial community structure (Leibold et al., 2010).

Common Prokaryotic Metabolisms

All prokaryotes need a carbon source and an energy source to survive, but utilize a variety of compounds or processes to fulfill those needs. Five basic physiological groups were defined by Ehrlich and Newman (2009); chemolithoautotrophs, photolithoautotrophs, mixotrophs, photoheterotrohps, and heterotrophs. These groupings allow prokaryotes to be organized based on where they obtain energy, and whether or not they are capable of assimilating inorganic carbon sources. The definition of these terms, and other used throughout this dissertation, are given in Table 1.1. No Archaea are known to be photolithoautotrophs, but all other groups include representatives from both Archaea and Bacteria.

In hydrothermal systems, anaerobic water with high concentrations of reduced metals typically discharges at spring and geyser features. These waters rapidly change in chemistry as atmospheric oxygen, decreased pressure, and cooler temperatures oxidize compounds and form precipitates. The resulting geochemical gradients are utilized by chemolithoautotrophic or mixotrophic microorganisms capable of using reduced sulfur compounds, metals, and other available compounds as energy sources. The rapid abiotic alteration of water chemistry, due to increased reaction rates at high temperatures, may limit life by making biologically catalyzed reactions less thermodynamically favorable (Amend & Shock, 2001). In this dissertation, I will focus on microorganisms with a methanogenic metabolism.

Term	Definition used in this dissertation	Source
Prokaryote	Term cytologically grouping Archaea and Bacteria together based on lack of membrane-bound organelles and nuclei	(Woese et al., 1990)
Population	All the individuals of one species in a given area at the same time	(Little et al., 2008)
Metabolic Guild	A group of species that occupy a common niche in a given community, characterized by exploitation of environmental resources in the same way	(Little et al., 2008)
Community	A multi-species assemblage in which organisms live together and interact in a contiguous environment	(Konopka, 2009)
Chemolithoautotroph	A category of organisms that oxidize inorganic compounds for energy, and assimilate carbon from inorganic carbon compounds	(Ehrlich & Newman, 2009)
Photolithoautotroph	A category of organisms that convert radiant energy from the sun to chemical energy, and assimilate carbon from inorganic carbon compounds	(Ehrlich & Newman, 2009)
Mixotroph	A category of organisms that derive energy from oxidizing reduced carbon compounds and/or inorganic compounds, and obtain carbon from organic carbon and/or CO_2	(Ehrlich & Newman, 2009)
Photoheterotroph	A category of organisms that convert radiant energy from the sun to chemical energy, and assimilate carbon from organic compounds	(Ehrlich & Newman, 2009)
Heterotroph	A category of organisms that derive energy from oxidizing organic compounds, assimilate carbon from organic compounds	(Ehrlich & Newman, 2009)

Table 1.1: Definitions of biological terms as used in this dissertation.

Methanogenesis

Methanogenesis is the biological production of methane as a metabolic by-product. Archaea is the only domain in which methanogenic representatives are known. All methanogens are obligate anaerobes, and they can be found in environments ranging from hyperthermophilic to psychrophilic (Nozhevnikova et al., 2003). Methanogens are associated with H_2-generating microorganisms, such as fermenters, because H_2 is used as

an electron donor in most methanogenic metabolisms. Methanogenesis is common in hydrothermal environments.

A limited number of substrates can be utilized by methanogens. One metabolic pathway includes the reduction of CO_2 to CH_4 using H_2 or formate as the electron donor. These hydrogenotrophic methanogens can therefore be heterotrophic (if they utilize formate) or chemolithoautotrophic (utilizing only H_2 and CO_2). In some cases, alcohols or CO may be used in place of CO_2 (Liu & Whitman, 2008). Two other methanogenic metabolisms are specific to the order Methanosarcinales; the reduction of methyl groups in methylated compounds using H_2 as the electron donor, or using acetate as a substrate by oxidizing the carboxyl group and reducing the methyl group (Liu & Whitman, 2008).

A number of the enzymes required for methanogenesis to occur are also unique to methanogenic Archaea. Coenzyme F420, for example, shuttles electrons from H_2 in place of ferredoxin, and has been shown to participate in numerous other redox reactions occurring within methanogenic cells (Jones et al., 1987). This coenzyme is useful in identifying methanogens microscopically, as well, because it autofluoresces blue-green under UV light, with maximum emission at 420nm (Doddema & Vogels, 1978).

Microbe-mineral interactions: Arsenic and Antimony

In this dissertation, microbe-mineral interactions will be limited to the known interactions of arsenic and antimony with prokaryotes, because these two toxic metalloids are present in high concentrations at the El Tatio Geyser Field, the field site for this study.

Arsenic (As) is common in many igneous rocks; therefore dissolved As is often found in hydrothermal waters. At Yellowstone National Park total As ranges from 10-40μM (Langner et al., 2001), around 70μM at Champange Pool in the Waiotapu hot springs of New Zealand, and 300-800μM at El Tatio Geyser Field in Chile (Landrum et al., 2009). The reduced form of As, the arsenite oxyanion (AsO_3^{3-}) is common in the anoxic discharge waters at spring and geyser features. Once exposed to sunlight and the atmosphere, arsenite species are oxidized to the arsenate oxyanion (AsO_4^{3-}) either by photooxidation, autooxidation, or microbial activity.

Microbial activity by both Archaea and Bacteria has been shown to oxidize, as well as reduce, As compounds (Ehrlich & Newman, 2009; Stolz et al., 2006), for both detoxification as well as a source of energy (Oremland, 2003). Both arsenate and arsenite are toxic to microorganisms. Arsenate interferes with oxidative phosphorylation, which inhibits ATP synthesis, and arsenite inhibits dehydrogenases and impairs the functions of many proteins. Several experiments have shown As(III) to be the more toxic species (Nakamuro & Sayato, 1981; Oremland, 2003; Sierra-Alvarez et al., 2004); despite this, some microorganisms convert As(V) into As(III) in order to pump it out of their cells. Because arsenate is similar to phosphate in structure, phosphate uptake systems sometimes allow arsenate to pass through the cell membrane. Once recognized as arsenate within the cell, it must be converted to arsenite in order to pump out the toxin without also removing phosphate from the cell (Stolz et al., 2006).

Arsenic occurs primarily in its reduced form, as As(III) in anaerobic waters. Within microbial mat communities, however, anaerobic microniches within mats allow

anaerobic microbial communities to live where As(V) is the dominant arsenic species. In anaerobic communities, As(V)-reducing prokaryotes compete with both methanogens and sulfate reducers. Methanogens may compete with As-reducers for H_2, which is used as an electron donor. The addition of a methanogen-specific inhibitor, 2-bromoethane-sulfonate (BES) to As(V)-exposed anaerobic communities has been shown to stimulate As(V) reduction (Field et al., 2004).

Toxicity experiments have shown that As(III) is more toxic to acetoclastic methanogens than hydrogenotrophic methanogens (Sierra-Alvarez et al., 2004). As(V) did not inhibit methanogenesis over short time periods (8-24 hours) even at the highest concentrations tested, around 500μM. However, metal-reducing prokaryotes found in anaerobic consortia reduce As(V) to As(III) over longer time scales, resulting in the complete inhibition of methanogenesis. This inhibition is slowed if As(III) concentrations are decreased through the precipitation of arsenic sulfide minerals such as realgar, which has been found at terrestrial hydrothermal systems such as the Yellowstone National Park and the Phlegrean fields of Italy (Nordstrom et al., 2005).

Antimony (Sb) shares some of arsenic's chemical and toxic properties (Bentley & Chasteen, 2002). Plasmids conferring arsenite resistance to bacteria have been shown to confer resistance to Sb(III) (Cervantes et al., 1994), and the mechanism for uptake and efflux of As(III) in *E. coli* is responsible for Sb(III) transport as well (Meng et al., 2004). Although it is not a particularly rare element, Sb typically occurs only in low concentrations in nature, and, thus, little is known about the affect and toxicity of Sb on microbial populations. Some microbial populations, including methanogens, have been

shown to produce methylated Sb (and As) gases, although laboratory experiments with thermophilic methanogens indicate that the heat rapidly destroys evidence of this volatilization (Meyer et al., 2008). In humans, Sb(III) has been shown to mitigate the mutagenic effects of As(III) (Gebel, 1998), and a link between microbial As toxicity and Sb may be possible.

Previous microbial studies involving antimony focus on biomethylation (Michalke et al., 2000), ties between arsenic and antimony resistance (Meng et al., 2004), and microbially-mediated oxidation-reduction reaction involving antimony. These studies were reviewed by Filella et al., (2007).

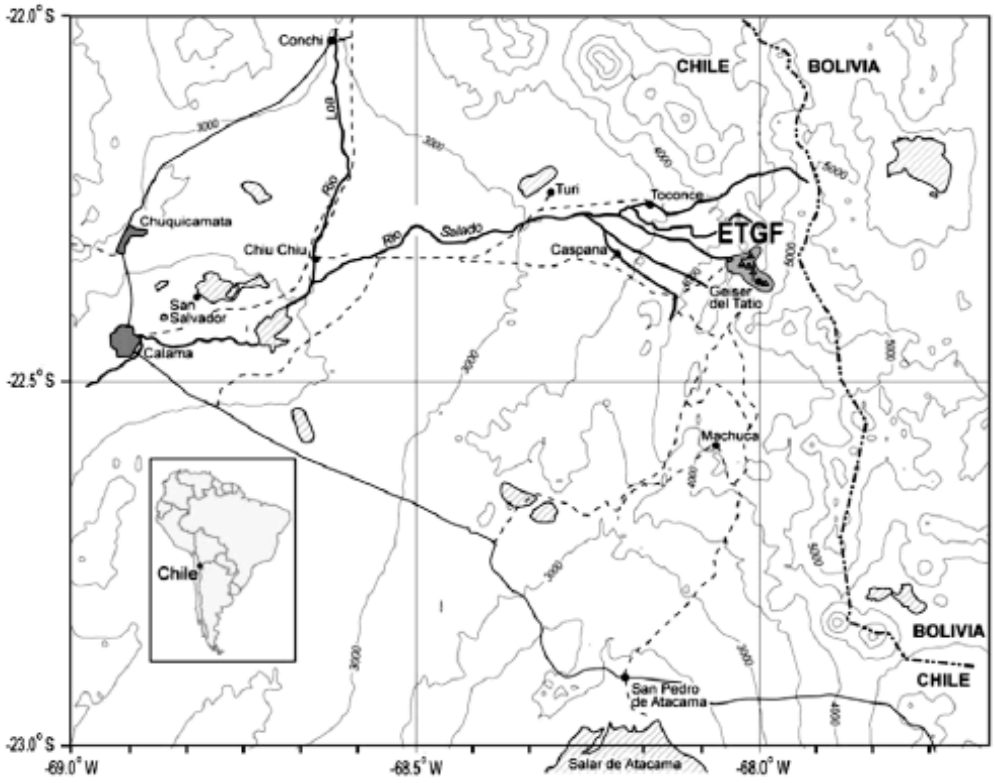


Figure 1.3: Map of Tatio, C.I. 500m (Landrum et al., 2009).

Carbon utilization and energy sources at ETGF

Prokaryotes that inhabit ETGF features were predicted to be primarily thermophilic and hyperthermophilic, neutrophilic, capable of tolerating saline environments, and resistant to heavy metals. We hypothesized that chemoheterotrophic prokaryotes would dominant metabolically, as little dissolved inorganic carbon (DIC) is available, and abundant reduced species are available.

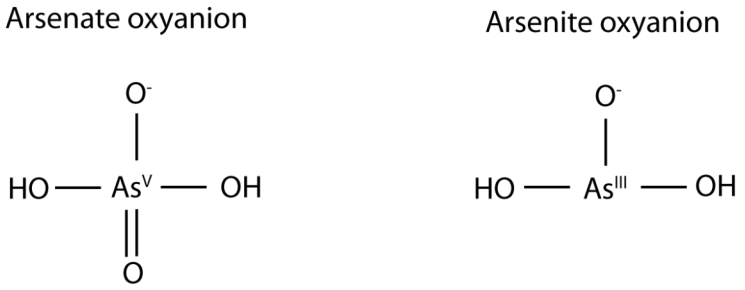
Carbon dioxide is a common constituent of hydrothermal gases, yet DIC is low in ETGF waters. Uptake by autotrophic microorganisms, as well as the lower partial pressure of carbon dioxide in the atmosphere at high elevations, may contribute to these low DIC concentrations, which range from 0.03 mM to 1 mM (2-66 ppm). In comparison, features of similar pH in Yellowstone National Park typically have concentrations between 2.5 mM and 120 mM DIC (Nordstrom et al., 2005).

Acetate and formate are used in many methanogenic metabolisms as heterotrophic carbon sources (Liu & Whitman, 2008); concentrations of both compounds were below detection limit at the ETGF sites tested. Organic carbon is likely supplied to heterotrophic microorganisms via decomposition of dead mat material, while autotrophic communities are likely to be carbon limited.

ETGF waters contain numerous compounds that can be utilized by chemolithotrophs and mixotrophs in microbial oxidation/reduction actions for energy production. Unusually, the most bioavaiable compounds are arsenic hydroxyanions (Figure 1.4), whereas at many hydrothermal sites sulfur compounds are the typical metabolites. Phototrophic Bacteria, such as cyanobacteria, are abundant in some

hydrothermal sites, and heterotrophs that obtain energy from the oxidation of organic carbon compounds are likely to be present, their abundance likely proportional to the number of autotrophs that can be supported by the DIC of the hydrothermal feature.

Figure 1.4: Hydroxyanions of arsenate and arsenite.



EL TATIO GEYSER FIELD SITE

Geological and Geographic setting

El Tatio is located near the Chilean-Bolivian border in the Antofagasta Province (Region II) of Chile (see Figure 1.3), at approximately 22.3°S latitude and 68°W longitude, and an altitude of ~4,300 meters. El Tatio is the largest geyser field in the Southern Hemisphere, and the 3rd largest in the world. Geothermal features are spread over a 30 square kilometer area, with most features concentrated in three major basins, designated the Upper, Middle, and Lower basins, which together cover approximately 10 square kilometers (Glennon & Pfaff, 2003). Discharge from all hydrothermal features forms the headwaters of the Rio Salado.

Environment and Water

ETGF in Northern Chile is one of the least-studied geothermal fields in the world, and is considered an ideal analogue system for early earth environments because of its unique geographical location and geochemical conditions. The combination of high altitude and low latitude results in high UV-A and UV-B radiation. ETGF is located within the hyper-arid Atacama Desert in an ecoregion in which few organisms are capable of surviving the extreme temperature fluctuations and altitude. Daily temperatures fluctuate over a wide range, and precipitation is less than 100 mm per year. High rates of evaporation increase the water salinity, which ranges from 95-175 mM, and concentrate dissolved hydrothermal metals; evaporation also contributes to the precipitation of salts and silica sinter.

Geologic and geothermal setting

El Tatio is part of the Altiplano-Puna Volcanic Complex, in the Central Volcanic Zone of the Andes. The surface features of ETGF are located on the downfaulted Tatio graben, and the geologic sequence underlying El Tatio includes (1) sedimentary rocks such as sandstones, shales, and conglomerates from the Middle and Upper Jurassic, (2) ignimbrites and andesites from volcanic episodes in the Late Tertiary to Late Miocene, (3) dacitic and rhyolitic ignimbrites from the Pliocene (Fernandez-Turiel et al., 2005). El Tatio has been a geothermal exploration site since the 1970s (Lahsen, 1988), and the Chilean government recently permitted further exploration by engineering companies (Lahsen et al., 2010). Indigenous groups, as well as environmental and economic (mostly

from the ecotourism community) interests raised concerns about the consequences geothermal energy projects will have on geyser flow, microbial life, and energy loss in the system.

Water chemistry

Hydrothermal waters discharge at geyser and spring features at temperatures ranging from ~60 to ~86˚C, the local boiling point. Water-rock interactions, absorption of magmatic steam, and dilution of thermal waters with meteoric water all play a part in dictating the isotopic and geochemical composition of ETGF waters (Giggenbach, 1978). The Na-Cl type waters are characterized by circum-neutral pH (6.5-7) and low inorganic carbon (0.1-0.5 mM TIC). Controls on water chemistry include the concentration of solutes by steam separation, the absorption of CO_2 and steam in meteoric waters, as well as the depth and residence time of hydrothermal waters (Giggenbach, 1978).

Dissolved solutes include high concentrations of sodium chloride, silica, and toxic metals including antimony and arsenic. Table 1.2 shows generalized water chemistry data for each of the three basins. Generally, the higher HCO_3^- values, lower Si, and lower As and Sb concentrations are indicative of more dilution with meteoric waters, indicating that the lower basin has the highest percentage of meteoric water mixed in. High concentrations of dissolved silica, originating in the underlying ignimbrites (Glennon & Pfaff, 2003), promote the formation of siliceous sinter at most hydrothermal features because cooling water precipitates amorphous silica.

Table 1.2: Water Chemistry (mM) of ETGF's 3 main basins.

Basin	pH	T(˚C)	[Cl]	[Na]	[B]	[K]	[Ca]	[Li]	[Si]	[As]	[Sb]	[HCO3-]
Upper	7.0	80.5	204	169	16.9	14.9	6.9	6.1	5.8	0.6	0.025	0.02
Middle	7.0	85.0	163	132	13.6	4.5	7.1	3.9	4.2	0.45	0.021	0.05
Lower	7.4	78.0	155	157	11.8	5.6	6.0	3.9	3.1	0.35	0.021	0.06

Antimony and arsenic are both toxic metalloids often found in hydrothermal fluids. At ETGF, the concentrations of As, ranging from 300-700 μM, and Sb, ranging in concentration from 8-25 μM, are extremely high and therefore play an important role both geochemically and biologically. Arsenic concentrations at ETGF are higher than at any other naturally occurring system, and arsenic acid is the primary pH buffer species in ETGF waters, buffering waters to a pH of 6.85 (see Equation 1.1).

Equation 1.1: Equation showing potential role of arsenic acid as a buffer in Tatio waters. Value at 80˚C from Landrum et al. (2009)

$$H_2AsO_4^- \leftrightarrow HAsO_4^{2-} + H^+ Ka^2 = \frac{\{HAsO_4^{2-}\}\{H^+\}}{\{H_2AsO_4^-\}} = 10^{-6.94}@25^oC; 10-6.85@80^oC$$

Microbial Mats

Microbial mats are present in many of the geothermal waters at ETGF. In addition to mat communities, planktonic microorganisms and cryptoendoliths living within silica sinter are present at hydrothermal sites, including fumaroles that emit vapor

but not water. Within hydrothermal waters, thick microbial mats are present in most areas where water temperatures do not exceed 70˚C; including the edges of boiling pools, run-off features from geysers and perpetual spouters, and spring features that discharge into stream water. Although hyperthermophiles live between 70 and 86˚C, no microbial mat communities are observed at ETGF at this temperature range, although planktonic microbes are likely present.

Thick (>2 cm) microbial mats at ETGF begin forming at ~70˚C. Similar temperatures are reported for other microbial mat communities (Jørgensen & Nelson, 1988; Sandbeck & Ward, 1982), the upper limit is defined in part by the upper temperature for photosynthesis (~73˚C) (Brock, 1985). Microbial mats are reported above 70˚C at terrestrial hydrothermal sites in the form of pink streamers (Reysenbach et al., 1994; Skirnisdottir et al., 2000), and in deep-sea marine hydrothermal sites (Davis & Moyer, 2008).

Figure 1.5: Streamers in El Tatio mats.

The color and thickness of microbial mat communities varies between hydrothermal features. Mat color is related to water temperature (Dunckel et al., 2009); in some cases, coloration corresponds to

differences in microbial mat community composition, in other cases, mat color is due to iron mineralization (Landrum et al., 2009). Many mats are coated with layers of precipitated amorphous silica. Vertical zonation of mat layers can be differentiated by color as well. In some areas, particularly ones in which mats are thicker than 5cm, trapped gas bubbles are present with microbial mats.

Some microbial mats contain streamers of mixed coloration, thickness and length, as well as a range of textures. These streamers are attached at their base to underlying mat, and are typically composed of a mixed microbial community. Streamers are often observed near source water and in high temperature areas.

The microbial community make-up of ETGF mats is not yet published, although 16S rRNA analyses were completed for bacterial diversity along temperature gradients of some features (Engel et al., submitted). No detailed report of microbial community structure and archaeal diversity at ETGF currently exists.

FEATURES OF INTEREST

Microbial mats analyzed for use in this dissertation were taken from features within the Middle and Upper Basins as well as an area designated as "Group M-III" (Glennon & Pfaff, 2003). Those features were originally chosen based on field measurements of dissolved methane, after gas chromatography indicated a disparate distribution of methane between geothermal features. Methane can be geothermally derived or biologically generated; the absence of dissolved methane near geyser and

spring sources, and its co-appearance with microbial mats at temperatures around 70˚C suggest microbial methanogenesis is responsible for dissolved concentrations.

Great Geyser (GG)

The Great Geyser is a well-characterized feature located in the middle basin at GPS coordinates -22.3˚S latitude and -68.0˚W longitude. This geyser discharges at local boiling and creates a discharge stream running ~75 meters long before emptying into a stream of mixed meteoric and hydrothermal origin. Waters cool rapidly with distance along the discharge transect, and red-orange mats with long, smooth streamers, start at a distance of ~20 meters downstream of the geyser, where water temperatures have cooled to ~70˚C.

At this site, analysis of mat composition indicates that little organic matter is actually present; instead, silica, iron and arsenic oxides dominate the mat. Mat thickness is approximately equal to the depth of the discharge waters; discharge ranges from approximately 7 to 2.5 cm thick. A silicified bench edges the run-off apron; however, silicification of the mat itself is limited to areas where mats are not fully

Figure 1.6: Great geyser mat.

submerged (Figure 1.6).

Middle Basin Springs (MBS)

The mixed hydrothermal and meteoric stream that converges with the Great Geyser discharge has several hydrothermal features discharging directly into it. Upstream of the confluence, two springs, designated here as the middle basin springs, are located directly across from each other feed into ~20˚C water (Figure 1.7). The stream is meteorically sourced, with hydrothermal inputs from upstream of the spring, including from within group M-III. Elevated salinity and metalloid concentrations are due to these hydrothermal inputs.

Microbial mats are not present in the stream prior to the MBS inputs. At the mouths of the springs and downstream, where hydrothermal and stream waters mix, microbial mats are abundant. The mats are structurally more complex than the GG mats, with a wide variety of colors, areas with and without (color) stratified mats, some areas with streamers, and some silicified mats. This suggests greater taxonomic diversity at this site.

Figure 1.7: Middle basin springs.

Near discharge points, silicification of mat material and surrounding rock/sediment is observed. Silicification is also evident on downstream microbial mats.

The stream water is approximately 75 cm wide and 5 cm deep where the two springs feed into it, but increases in depth to approximately 20 cm deep just after the spring input. Spring water exits features between 65 and 75˚C. No significant gas discharge is seen at these features. The waters exiting the springs have markedly different chemical compositions, including differences in pH and sulfate.

Upper Basin Pool (UBP)

This site is a hydrothermal, but relatively low-temperature, pool located in the upper basin of ETGF. Mats here are thick and uniformly pink-brown in color; sediments underlying the mats are light brown but include some areas with bright yellow precipitates, thought to be Sb or As minerals. Water temperature is 33˚C, and the depth of the pool has not been measured. Samples were taken in a relatively shallow area near the edge of the pool, approximately 10 cm in depth.

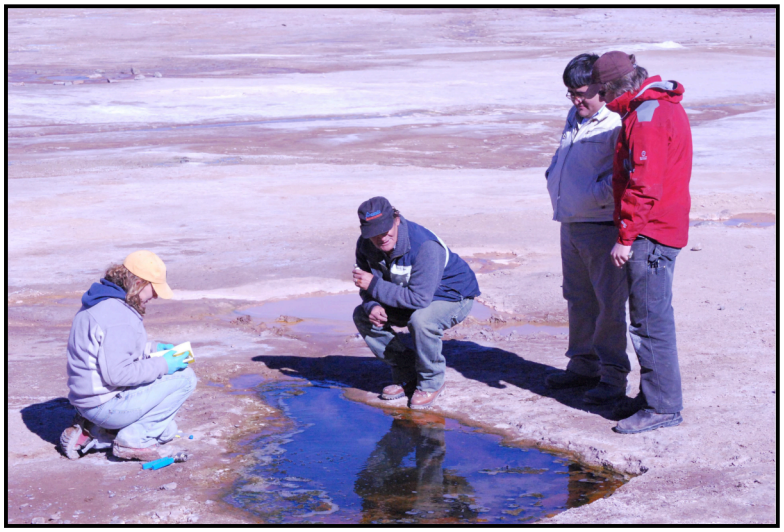


Figure 1.8: Upper Basin Pool.

Low temperature stream (LTS)

In order to compare the low temperature pool to another mesophilic site, a stream of mixed hydrothermal and meteoric waters located in the M-III group of features was sampled. Dissolved methane concentrations were not measured at this site. This site falls within headwaters of the Middle Basin Stream, and is approximately 2km upstream of the MBS springs. This group of features consist mostly of mud pots, and, unlike other sites, has abundant grasses growing adjacent to hydrothermal features. Water chemistry, including the low concentrations of silica and arsenic at this site, indicates hydrothermal features in this area contain steam-heated, near-surface meteoric waters.

Figure 1.9: Low Temperature Stream.

SAFETY CONCERNS

The isolation and difficulty accessing ETGF brings safety concerns into the forefront of all work done there. El Tatio is a 1.5-hour drive and a two thousand meter elevation climb from San Pedro de Atacama, my home base while doing fieldwork. The altitude of San Pedro de Atacama is around 2,400 meters, and some acclimatization there

has been found beneficial before climbing to the over 4,200 meter elevation of ETGF. The nearest airport is 60 miles away, in the city of Calama. The road and signage between San Pedro and ETGF was improved in recent years due to the influx of geothermal engineers in this area, as well as attempts to improve ETGF's appeal as a tourist attraction.

No cell phone service is available in the area. Bathrooms are now available at the welcome center for the geyser field; however, fuel, water, food, and other necessities are not available and sufficient quantities of each should be brought on each trip. High altitudes increase the chance of dehydration, and the arid climate of this region compounds this affliction. Additionally, the altitude combined with the low latitude makes sunburn likely; wearing long sleeves, a hat, and sunscreen is recommended. Some people experience nausea, headaches, or other symptoms of altitude sickness.

RESEARCH QUESTIONS AND HYPOTHESES

Do novel archaeal taxa inhabit ETGF?

Archaea are taxonomically and physiologically diverse, and while great advances have been made in cataloging the presence of Archaea in a variety of environments, the list of archaeal taxa is not exhaustive at this point. The two definitive phyla of Archaea, the Euryarchaeota and Crenarchaeota, are more evolutionarily distant from each other than bacterial phyla are. (Forterre et al., 2007). This suggests many archaeal phyla are as yet undiscovered, and that the diversity we find may cover a great deal of evolutionary distance.

Although any environment could host numerous Archaea, high-temperature environments are more likely to contain hyperthermophilic and thermophilic Archaea, which may retain characteristics of the last common archaeal ancestor. The relative isolation of ETGF, the limited interaction microbial mat communities there have with eukaryotic organisms, and the extreme conditions at ETGF, including high water temperatures, makes ETGF a good analogue for proposed ecosystems of ancestral Archaea.

Many hyperthermophilic Archaea have sulfur-based metabolisms (Kletzin, 2007). The relatively low sulfur concentrations at ETGF suggest Archaea found there may use atypical metabolic pathways; or that Archaea unable to compete with sulfur-utilizing organisms may be present there. The low sulfur at ETGF may also affect other metabolic guilds of microorganisms, because sulfate reducers often compete with methanogens for hydrogen (Lovley et al., 1982).

For these reasons, I hypothesized that novel archaeal taxa would be found at ETGF, and that some of those taxa would be within groups of methanogenic Archaea. My research approach for testing this hypothesis can be seen in Chapter 2 of this dissertation.

How does archaeal community composition vary between sites?

In determining the archaeal community diversity at ETGF, the genetic data from microbes collected from a number of hydrothermal features at the geyser field were compared to genetic information compiled in GenBank and the greengenes database

(DeSantis et al., 2006). The evolutionary distance between ETGF environmental samples and sequences in those databases was calculated to determine how closely related Archaea from ETGF are to both to cultured Archaea, whose metabolisms and optimal growth conditions have been tested in a laboratory setting, and uncultured Archaea, which have been isolated from environmental samples.

Looking at the species richness, or, in this case, the OTU richness, of the community as a whole contributes to our understanding of how similar ETGF community structure is to other hydrothermal systems, but gives no information about the similarity of communities between hydrothermal features within ETGF.

I hypothesized that a great deal of phylogenetic diversity would be present between sites at ETGF, because differences in water chemistry, dissolved gas composition, and temperature all indicate that substrates for microbial metabolism vary between sites. The difference in community composition between sites or along environmental gradients is called the beta diversity of the system, and takes both OTU richness and the number of overlapping OTUs between two sites into account. The second and fourth chapters will both discuss beta diversity analyses at ETGF, the fourth chapter addressing the topic more fully.

What physicochemical parameters affect archaeal and bacterial diversity?

In recent years, the importance of Archaea in metabolic cycling of nutrients within many ecosystems was documented (Caffrey et al., 2007; Leininger et al., 2006; Nicol & Schleper, 2006). In typical phylogenetic analyses of microbial ecosystems,

bacterial and archaeal sequences are analyzed together as one microbial community. The diversity of the system is determined using both bacterial and archaeal OTUs. I hypothesized that due to the different metabolic niches taken up by Bacteria and Archaea, the beta diversity (including the number of overlapping OTUs between hydrothermal sites) would differ for Bacteria and Archaea. Additionally, I hypothesized that the physicochemical parameters dictating the community composition and diversity for Archaea and Bacteria will differ. In Chapter 2 of this dissertation I discuss differences in bacterial and archaeal diversity between geochemically distinct sites, and show how those differences correlate to physicochemical parameters.

How do arsenic and antimony toxicity affect Archaea at ETGF?

Both As and Sb are known toxins, and many microorganisms have detoxification mechanisms or other methods of coping with high concentrations of these metalloids (Filella et al., 2007; Oremland, 2003). The oxidation state of the As is important in determining toxicity, because As(III) and As(V) have different toxic effects on microorganisms. At ETGF, concentrations of both As and Sb are very high. I hypothesized that methanogens at ETGF have the capacity to detoxify As, or are found in communities in which As-oxidizers are present. Additionally, I hypothesized that the presence of Sb would alter the toxicity of As, because the uptake and resistance mechanisms for Sb and As are similar for many organisms (Lehr et al., 2007; Meng et al., 2004; Silver et al., 1981).

Archaea at ETGF

El Tatio is a unique environment in which to study Archaea. The physicochemical conditions and extreme isolation provide a natural laboratory wherein nutrient cycling is predominantly controlled by microorganisms, and water chemistry is dictated only by hyrdrothermal inputs and microbial transformations. My goal in focusing on Archaea at the El Tatio Geyser Field is not only to expand knowledge of genetic diversity with extant Archaea, but also to explore phylogenetic groups with respect to water chemistry, and diversity as a function of environmental controls.

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Chapter 2: The Distribution and Diversity of Methanogens and Other Archaea at El Tatio Geyser Field

ABSTRACT

Extremely high measured concentrations of arsenic at the El Tatio Geyser Field (ETGF) of northern Chile suggest that this location may host novel microorganisms, including Archaea. Furthermore, the detection of methane in only a few geyser features points to the possibility that methanogenic Archaea, and therefore other archaeal groups, may not be ubiquitous in ETGF waters, but instead may vary between features with different geochemical compositions and temperatures. MPN analyses support the biogenicity of the methane, as does 16S rRNA analysis, although isotopic analyses are inconclusive. Results from four sites containing dissolved methane show that archaeal communities are diverse, and suggest novel archaeal taxa inhabit these waters. Archaeal community composition shows correlations to water temperature and geochemical parameters: Crenarchaea are most abundant at sites with higher temperatures and salinities, and are dominated by *Desulfurococcales* representatives. Abundance of methanogens is low in waters with low dissolved inorganic carbon concentrations, and the phylogenetic affiliation of methanogens varies between sites. Even sequences from geochemically distinct sites grouped together phylogenetically, and thermophilic Archaea, including Crenarchaea, were found at lower temperature sites.

INTRODUCTION

Microbial mat communities in extreme environments such as hot springs allow metabolically diverse microorganisms to thrive, despite physicochemical challenges. Many studies have focused on the diversity and distribution of chemolithoautotrophic (Spear et al., 2005) or phototrophic (Doemel & Brock, 1977; Ferris et al., 2005; Paerl et al., 2000) microbial populations from aquatic extreme environments. Archaea are often incorporated into prokaryotic diversity studies (Allen et al., 2009; Sørensen et al., 2005), and recent research suggests they are important in nutrient cycling, even when archaeal biomass makes up a small fraction of the total biomass (Mehta & Baross, 2006; Prosser & Nicol, 2008). In hot spring environments, the importance of Archaea in microbial ecosystems may be even more significant because of the limited presence of eukaryotic organisms, and the capacity of Archaea to tolerate extreme environmental conditions.

Research in recent years has greatly advanced our knowledge of Archaea, including their unique genetic traits and metabolic abilities. Methanogenesis, limited to groups within the Euryarchaeota, is a well-characterized metabolic pathway (Liu & Whitman, 2008) unique to the domain Archaea that can play an important role in the geochemistry and carbon cycling of extreme environments (Sandbeck & Ward, 1982; Ward, 1978; Zeikus et al., 1980). Methane-producing Archaea, or methanogens, are phylogenetically diverse and geographically widespread. The presence of thermophilic and hyperthermophilic methanogens is well documented in deep sea hydrothermal systems (Jeanthon et al., 1999; L'Haridon et al., 2003; Wagner & Wiegel, 2008), but methanogen

populations are not well represented in many terrestrial hydrothermal features, such as those at Yellowstone National Park (Barns et al., 1996; Barns et al., 1994), New Zealand's Waiotapo geothermal region (Hetzer et al., 2007), and in southwestern Iceland (Skirnisdottir et al., 2000). This absence is not indicative of low methanogenic populations as much as the temperature range sampled. In terrestrial hot springs, methanogenesis is more often found to occur between 50 and 60˚C (Sandbeck & Ward, 1982; Ward, 1978), although they have been isolated at higher temperatures (Stetter et al., 1981). As most studies of archaeal diversity within terrestrial hot spring systems focus on higher temperature mat communities (65-93˚ C), methanogens from these settings have not been well characterized.

Previously, the methanogen *Methanofollis tationis* was isolated from mud pots at ETGF, with an optimal growth temperature of 37-40˚C (Zabel et al., 1984). From our preliminary work, dissolved methane concentrations up to 550 μM have been measured from thermal features ranging from 30 to 71˚C. While earlier work at Yellowstone National Park shows that these temperatures are within the range of known methanogenesis (Bryanskaya et al., 2006; Sandbeck & Ward, 1982; Ward, 1978), we considered that the extremely high As(III) concentrations could adversely affect the distribution and rate of methanogenesis. A myriad of other environmental factors may also challenge methanogens and other microorganisms, such as the low dissolved inorganic carbon (DIC) concentration.

In this study, 16S rRNA analysis and other techniques to determine the archaeal community composition at high (~65˚C) and low (~35˚C) temperature hydrothermal

features at the El Tatio Geyser Field in northern Chile. The goals of this study were to describe the composition of archaeal communities at the various hydrothermal features, and to evaluate the extent of methanogenesis at these features. The findings, which are the first to describe Archaea from ETGF, uncover additional diversity of some archaeal groups in thermal settings, and also expand our understanding of methanogenic activity in hydrothermal habitats.

MATERIALS AND METHODS

Study location

ETGF is the third largest geyser field in the world at approximately 10 km^2 and having >100 hydrothermal features (Glennon & Pfaff, 2003) (Figure 2.1). Despite the presence of large microbial mats at many features, no studies characterizing microbial populations at ETGF have been published to date. ETGF is located in the Andes mountains at an elevation of ~4300 m, and experiences high solar UV radiation (Phoenix et al., 2006), and an arid climate. The high altitude and low latitude (22˚S) causes variations in annual and diurnal air temperatures and high evaporation rates that concentrate hydrothermal metalloids, such as arsenic, in geyser discharge waters. Arsenic concentrations range from 300 to 700 μM, with ~80% found as reduced arsenite [As(III)] in the discharge springs (Landrum et al., 2009).

Most features, which include boiling pools, mud pots, geysers and hot springs, discharge silica-rich, saline water at circumneutral pH and temperatures at or near the

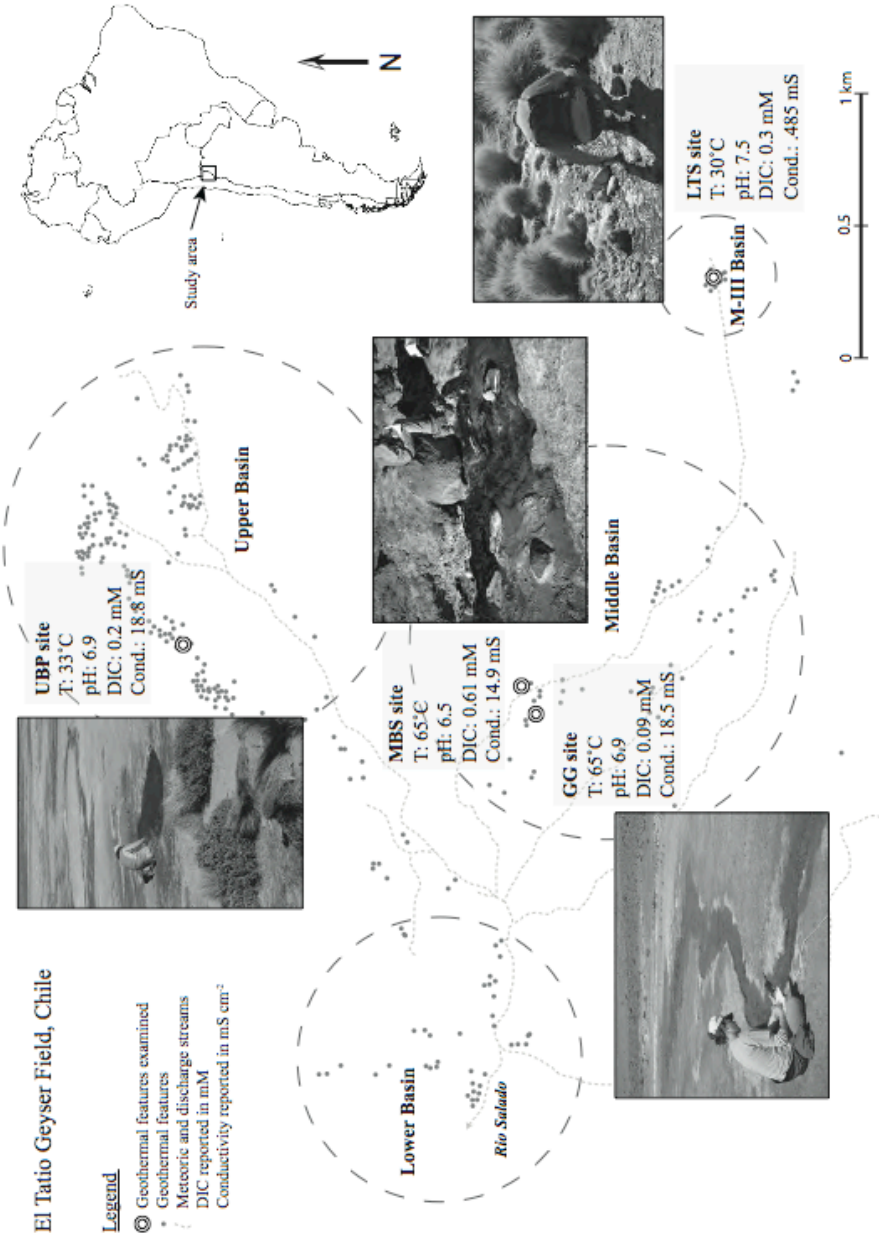
local boiling point, ~85˚C. Features are concentrated primarily in three major basins, designated by Glennon and Pfaff (2003) as the upper, middle and lower basins, and their terminology is followed here. Our study includes features from the upper and middle basins, as well as from group M-III (Glennon & Pfaff, 2003), a small group of mud pots and hot springs. The geyser waters flow into small streams that form the headwaters of the Rio Salado, an important source of drinking and irrigation waters for local inhabitants (Landrum et al., 2009).

Sampling locations and sample collection

Four sites were selected for this study based on preliminary analyses of >25 individual spring sites (Landrum et al., 2009; Phoenix et al., 2006) (Figure 2.1): two sites at ~65˚C from the Middle Basin, 30 m downstream of the Great Geyser outlet (GG) and a second in a mixing zone where a hot spring discharges into cool stream water (MBS) that originates from a mixture of meteoric and upstream spring water within the M-III group, one site at ~35˚ C from the headwaters of the Middle Basin Stream within the M-III group (LTS), and one site at ~35˚C from a discharge pool in the Upper Basin (UBP). Water and microbial samples were taken in December of 2006. One bulk mat sample was taken per site, and used for all microbial analyses.

Unstable parameters including water temperature, pH, dissolved oxygen, and total dissolved solids (TDS) were determined in the field by standard electrode methods. Dissolved ammonia, sulfide, and ferrous iron were measured in the field by colorimetry (Chemetrics Inc., Calverton, VA, USA).

Figure 2.1: Map of sample locations and basic geochemical parameters at MBS: the middle basin stream site, GG: the great geyser site, LTS: the low temperature stream site, and UBP: the upper basin pool site.



Water samples filtered to 0.2 μm were collected for analysis of dissolved anions, metals, and dissolved inorganic carbon (DIC). Anions were determined using ion chromatography, and dissolved metals were analyzed from nitric acid-preserved samples on an Agilent 7500ce ICP-MS. DIC was determined by NDIR detector using an Apollo 9000 Combustion TOC Analyzer (Teledyne Tekmar, Mason OH, USA). Bulk mat samples were collected aseptically in sterile tubes with native water, and transported and stored at 4˚C until analysis, which was no more than two weeks after collection.

Dissolved gas measurements

Dissolved CH_4 was determined in the field by gas chromatography. One water sample was collected from each site. Samples were collected without headspace in 60 mL serum bottles and crimp-sealed with butyl rubber caps and aluminum seals. Within 8 hours of sample collection, ~20 mL of water were displaced by injecting methane-free ultra-high purity H_2 into the bottles to create an all-hydrogen headspace. The samples were allowed to equilibrate for ~1 hour, during which equilibration was encouraged by periodic shaking, before headspace gas was sampled and analyzed using a SRI 310 gas chromatograph (GC) (SRI, Torrance CA, USA) with a ShinCarbon ST 100/120 column and H_2 carrier gas. CH_4 concentrations greater than atmospheric were counted as CH_4-positive; those samples with less than atmospheric CH_4 were CH_4-negative. Total dissolved CH_4 was quantified assuming that the headspace CH_4 was in equilibrium with the water and using a Henry's law constant of 1.5×10^{-3} $M \cdot atm^{-1}$ (Hine & Mookerjee, 1975).

Dissolved H_2 was determined on separately collected samples using the bubble strip method (Wiedemeier et al., 1998), whereby geyser fluids were pumped at a constant rate through a 250 mL flask with both a water outlet and an initial trapped air bubble. Each flowing sample was allowed to equilibrate for 30-60 minutes depending on the temperature of the water (i.e. more time for cooler temperatures), after which the gas bubble was extracted and injected into an evacuated 30 mL serum bottle with a blue butyl rubber stopper (BellCo Glass Inc., Vineland NJ, USA) and crimped aluminum cap. Samples were analyzed within two weeks of collection. Dissolved H_2 concentrations were calculated using temperature-corrected Henry's constant: $C_w=C_gH_c$, where C_w is the concentration of H_2 in water, C_g the concentration of H_2 in the gas, and H_c is Henry's law constant. The equation: $H_c=P_{H2}/RTC_w$, used for temperature-correction of Henry's law constant in Spear et al. (2005) was used to estimate H_c. The temperature, T, is the measured bubble temperature (K) and R is 0.0821 liters·atm·mol^{-1} K^{-1}. Values for C_w and P_{H2} were adjusted for a total atmospheric pressure of 0.605 atm, so that $P_{H2}=10^{-5.13}$ and $C_w=10^{-8.24}$.

MPN cultures and analysis

Most Probable Number (MPN) analysis (Bennett et al., 2006) was used to quantify viable culturable methanogenic biomass in selected microbial mat samples. A dilution salts medium was prepared as described by Bekins et al. (Bekins et al., 1999), which was pre-reduced and anaerobically sterilized (PRAS) according to Holdeman and Moore (1972). Two different growth media were used per sample: one was amended

with 18 mM sodium acetate to enumerate acetoclastic methanogens, and the other with a 140 kPa overpressure of 30% CO_2 and 70% H_2 to enumerate hydrogenotrophic methanogens. Acetate-amended media were gassed with H_2 to maintain positive pressure. Enumeration was completed in 10 mL glass serum bottles filled with 5 mL of PRAS media and aseptically inoculated in a Coy anaerobic chamber with an atmosphere of 1.5% H_2 in N_2 and CO_2 (Coy Laboratory Products Inc., Grass Lake MI, USA). Mat-water slurries were created by physically disaggregating mat material, and 1 gram (wet weight) of this mat-water slurry was used to inoculate the first bottle in each dilution series. Five ten-fold serial dilutions were done for each sample and medium. Two ten-fold serial dilutions of sterile water were completed for each media as a negative control and to obtain background methane concentrations. After a 3-month incubation period at room temperature, bottles were scored for positive growth by analyzing the headspace of each bottle for CH_4 by GC. The dilution factor and number of positive scores was entered into the MPN Calculator program (Build 2.0; M. Curiale, http://members.ync.net/mcuriale/mpn/index.html), which estimated the MPN of cells per gram of mat sample. Following analysis, the most dilute CH_4-postive bottles from the MBS site were transferred into 60 mL serum bottles containing the same growth medium, gassed with a 140 kPa overpressure of 30% CO_2 and 70% H_2, and incubated anaerobically at 65°C.

Based on observed fluorescence (Doddema & Vogels, 1978), two MPN cultures were chosen for DNA extraction, clone library construction, and sequencing.

Stable carbon isotopes of dissolved inorganic carbon and methane

The $\delta^{13}C_{DIC}$ was analyzed at three different sites: a sample from the GG site, near the source, a sample from the MBS springs where mat samples were analyzed, and a sample from the cool waters upstream of the MBS springs (Table 2.2). Methane $\delta^{13}C$ values were measured for four sites, one each from the GG, MBS, UBP, and LTS sites.

Unfiltered water samples were collected in 1-liter nalgene bottles with no headspace, and sealed to prevent exchange with atmospheric gases. DIC was preserved for $\delta^{13}C$ analysis by adding ammoniacal strontium chloride, which precipitates strontium carbonate. The $^{13}C/^{12}C$ values of the carbonate were determined by Coastal Science Laboratories, Inc. (Austin, TX).

Unfiltered water samples for $\delta^{13}C$ CH_4 analysis were collected into pre-evacuated 120 mL serum bottles preserved with mercuric chloride to prevent biological methane production between sample collection and analysis. 60 mL of sample water were added to each bottle, and helium was added to the headspace to ensure pressure inside the bottle with greater than atmospheric. Headspace gas was assumed to be in equilibrium with dissolved gases, and appreciable fractionation between aqueous and gaseous CH_4 does not occur (Fuex, 1980). Headspace gas was injected into a helium carrier line that passed through a liquid nitrogen trap to remove CO_2, then over copper oxide at 800˚C to oxidize CH_4 to CO_2. The gas sample was then cryofocused in a second liquid N_2 trap to concentrate the CO_2 derived from CH_4 before analysis. Samples were analyzed for $^{13}C/^{12}C$ in a ThermoFinnigan Delta V Plus isotope ratio mass spectrometer (ThermoFinnigan Inc., Bremen, Germany).

DNA extraction and PCR amplification of rRNA genes

Total environmental DNA from 1 mL aliquots of mat-water slurries was extracted from from the four sites, as well as from two MPN enrichment cultures from the MBS site. Extractions were done using a soil DNA extraction kit (Mo-Bio Laboratories Inc., Carlsbad CA, USA), and final DNA concentrations were between 13-50 ng·μl^{-1}, as determined spectroscopically (Nanodrop, Thermo Fisher Scientific Inc., Waltham MA, USA). 16S rRNA gene sequences were amplified by PCR using the universal archaeal primers UA571F (5'-GCYTAAAGSRICCGTAGC-3') and UA1204R (5'-TTMGGGGCATRCIKACCT-3') (Baker et al., 2003), as well as archaeal primers UA333F (5'-TCCAGGCCCTACGGG-3') and UA958R (5'-YCCGGCGTTGAVTCCAATT-3') (Reysenbach & Pace, 1995). Both sets of primers were used for the MBS and UBP sites; and only the UA333F/UA958R primer pair was used for the GG and LTS sites. The primer pair UA571F/UA1204R utilizes a region conservative within 2 known and 2 proposed archaeal phyla: Euryarchaea, Crenarchaea, Korarchaea and Nanoarchaea (Baker & Cowan, 2004). Each reaction contained 2-5 μl DNA, and concentrations of each component were: 1x PCR buffer, 3.5 mM $MgCl_2$, 8 $\mu g \cdot mL^{-1}$ BSA, 0.04 $U \cdot \mu L^{-1}$ Taq polymerase, 0.5 mM dNTPs, and 0.25 mM each primer. PCR conditions were 94˚C for 2 min, 30 cycles of 94˚C for 1 min, 60˚C for 1 min, 72˚C for 1 min, and a final 10 min extension at 72˚C. At least two PCR amplifications were

completed for all samples. PCR products were visualized on 1.7% TAE agarose gels containing 1.5x SYBR Safe dye (Invitrogen Inc., Camarillo CA, USA).

Cloning and sequencing

PCR products of the correct size were separated by agarose gel electrophoresis, recovered from the gel using a freeze-squeeze method (Thuring et al., 1975), and cloned with a pCRII-TOPO TA cloning kit with One Shot TOP10F' chemically-competent cells (Invitrogen Inc.). No unusually long bands indicative of introns (Takai & Horikoshi, 1999) were present. Plasmids were extracted using an alkaline miniprep method (Ausubel et al., 1990); samples were screened by restriction length polymorphism analysis (RFLP) and selected clones representing unique RFLP patterns were sequenced in both directions using an AB3730 capillary sequencer at the University of Texas at Austin's DNA Sequencing Facility.

Sequence analyses

Sequences were compared to known archaeal sequences in the GenBank database using BLAST (Altschul et al., 1990). Sequences were aligned using the NAST sequences aligner (DeSantis et al., 2006) and checked for chimera using Bellerophon (Huber et al., 2004). Five chimeric sequences were found and removed from further analysis. Chimera-checked sequences were inserted into ARB (Ludwig et al., 2004), and aligned sequences over 450 bp in length were used in tree construction. Operational taxonomic units (OTUs) were identified using mothur (Schloss et al., 2009). OTUs were identified

at the 95 and 90% similarity cut-off. A 90% similarity as used to choose sequences from each site to include in the phylogenetic tree, which were exported along with archaeal taxa from the greengenes database (DeSantis et al, 2006) into RAxML version 7.0.4 (Stamatakis, 2006). A maximum likelihood tree was created using the model of evolution GTR+I+G, as determined by the program Modeltest version 3.7 (Posada & Crandall, 1998).

EstimateS (version 8.20) <http://viceroy.eeb.uconn.edu/ estimates> was used to compute a range of richness estimators for abundance, diversity, and incidence data from clone libraries (Colwell, 2009). OTUs defined by mothur at the ≥97% similarity level were used to define species level taxonomic affinities and to determine diversity indices, including the Chao1 and Abundance-base Coverage Estimator (ACE) for OTU (i.e., "species") richness and the Shannon-Wiener (H') and Simpson's (D) indices for OTU abundance and evenness, respectively. The sequences used in this study can be found in GenBank under the accession numbers HQ636626 – HQ637175.

RESULTS AND DISCUSSION

Aqueous- and gas-phase geochemistry

All geyser fluids had similar measured pH, and GG, MBS, UBP had similar concentrations of Na^+, ΣAs, ΣSb and Cl^- (Table 1), which were higher than the measured values from LTS because its composition likely reflects being a mixture of freshwater runoff with geyser discharge waters. As such, the four sites were categorized based on

temperatures and salinity: (i) low-temperature dilute fluids (LTS); (ii) low-temperature saline fluids (UBP); and (iii) hot, saline fluids (GG and MBS).

All sites examined were CH_4-positive, with the highest concentrations in the lowest temperature fluids. H_2 values varied with hydrothermal features, but over concentrations were higher than those seen in many hydrothermal features at Yellowstone, (Spear et al., 2005). MBS and UBP had similar dissolved H_2 concentrations, and H_2 was not measured at LTS. DIC, SO_4^{2-} and H_2 were lowest at GG. In the four sites included in this study, arsenic and antimony concentrations increase as salinity increases. In general, H_2 values did not correlate to CH_4, but did increase with increasing DIC concentrations. Interestingly, CH_4 concentrations were less than atmospheric in the source waters at the GG site that discharges at ~85˚C (local boiling), but CH_4 was detectable downstream of the GG outflow after temperatures fall below 65ºC (Table 2.1).

Isotopic data

The three samples tested were enriched in $\delta^{13}C_{DIC}$, with values ranging from -5.0 to +8.8‰. These heavy DIC values agree with previous studies showing that the $\delta^{13}C_{DIC}$ at an ETGF fumarole was -8.85‰ (Tassi et al., 2005). The heaviest $\delta^{13}C_{DIC}$ was not near a hydrothermal source, but upstream of the MBS springs. The water at this site is a mixture of meteoric and cooled hydrothermal discharge (see Table 1 for water chemistry). While it is free of microbial mats, it is downstream of both hydrothermal

Table 2.1: Water chemistry analysis, MBS: middle basin stream site, GG: great geyser site, LTS: low temperature stream site, UBP: upper basin pool site.

Site	Feature	Mat Type	T °C	pH	DIC mM	Conductivity mS	As mM	Sb µM	Na^+ mM	Cl^- mM	SO_4^{2-} mM	CH_4 µM	H_2 nM
GG	Pool of geyser source water	No mat	82	6.9	0.11	18.6	0.44	19	156	175	4.5	bdl	333
GG	Geyser discharge, 33 m downstream from outlet	Orange streamers	65	6.9	0.09	18.5	0.46	21	160	180	3.2	13.3	67.1
MBS	Upstream of springs	No mat	24	8.0	0.10	16.37	0.42	15	158	148	2.6	bdl	100
MBS	Spring discharging into stream	Cyanobacterial mat	65	6.5	0.61	14.9	0.28	9	93	90	2.3	19.1	363
LTS	Cool fresh stream upstream of MBS	Cyanobacterial mat	30	7.5	0.3	0.49	0.001	0.25	3	3.1	2.1	181	185
UPB	Discharge pool	Thick orange and pink mat	33	6.9	0.2	18.8	0.48	14	150	150	8.8	278	154

features and microbial mats. The extremely enriched $\delta^{13}C_{DIC}$ may indicate that isotopically light DIC was already stripped, leaving a ^{13}C-enriched pool of DIC behind.

The $\delta^{13}C_{CH4}$ values are heavy, as well, and are atypical of CH_4 produced by methanogens. The extremely enriched $\delta^{13}C_{CH4}$ observed suggests mature themogenic gases could be responsible for both the observed DIC and CH_4 (Whiticar, 1999).

The highest $\delta^{13}C_{CH4}$ measured was at the UBP site, with a value of -15.3‰. Both cyanobacterial mats (at the MBS and LTS sites) had delta values of ~ -5‰, and the source water for the GG stream had a delta value of -6.6‰. The depletion observed in the UBP mats may be indicative of microbial activity, despite $\delta^{13}C_{CH4}$ values more enriched than those typical of methanogenesis.

The per mil range of biogenic CH_4, as well as the isotopic fractionation that occurs during the metabolism of CH_4, depends on the metabolic pathway and carbon source utilized by the methanogens. Hydrogenotrophic methanogenesis results in $\delta^{13}C_{CH4}$ values of -110 to -60‰, and acetoclastic methanogenesis results in more moderate fractionation, between -65 and -50‰ (Whiticar & Faber, 1986). However, these fractionations and ranges do not hold true in closed systems, where CO_2 limitation occurs.

ETGF is a carbon limited system, and the carbon that is available is isotopically heavy. Biogenic production of isotopically-heavy CH_4 has been observed in carbon-limited systems (Whiticar, 1999), particularly those in which alkaline pH limit the amount of dissolved CO_2 (Bradley, 2008). In addition, high concentrations of H_2 can decrease isotopic fractionation (Takai et al., 2008; Valentine et al., 2004); and

concentrations of H_2 at the ETGF sites correspond to high-H_2 sites at Yellowstone (Spear et al., 2005).

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Table 2.2 $\delta^{13}C$ analysis of DIC and CH_4 at select hydrothermal sites

Site	Feature	Mat Type	‰ $\delta^{13}C_{DIC}$	‰ $\delta^{13}C_{CH4}$
GG	Pool of geyser source water	No mat	-3.5	-6.6
GG	Geyser discharge, 33 m downstream from outlet	Orange streamers	nd	nd
MBS	Upstream of springs	No mat	+8.8	nd
MBS	Spring discharging into stream	Cyanobacterial mat	-5.0	-5.3
LTS	Cool fresh stream upstream of MBS	Cyanobacterial mat	nd	-5.1
UPB	Discharge pool	Thick orange and pink mat	nd	-15.3

Methanogenic MPN analyses and archaeal diversity

Methanogens were enumerated from three sites (Table 2.3). The highest MPN value for all media was from the 65˚C MBS site, which had low dissolved CH_4 measured in the field and the highest field-measured H_2. The UBP and GG sites, the former at 33˚C and the latter at 65˚C, had lower MPN values for all media.

If field methane concentrations are indicative of biogenic methane production, methanogens should be most abundant at the UBP site. This was not observed, however;

indicating either that MPN results do not accurately depict field abundance of methanogens, or that measured field methane is not all biogenic, and therefore not indicative of the population size for methanogens.

Diversity values (Table 2.4) indicate that the diversity varies greatly between sites. The MBS site is the most diverse, which suggests a more complex microbial community.

Cultures grown at room temperature incubation may have prevented the growth and metabolism of thermophilic methanogens, thereby underestimating the number of cells/gram at high temperature sites. Similarly, lack of methanogens in MPN cultures from the GG site may not indicate their absence, but could suggest that methanogens at this site are not culturable in these conditions. Despite these drawbacks, phylogenetic analyses of environmental samples correlate to MPN data, to suggest that methanogens are present at the UBP and MBS sites, but very few are present at the GG site.

Table 2.3 MPN enumeration results, estimated number of methanogenic cells per gram mat material, for three sample sites and each carbon source tested.

Site	Estimated cells/g (wet weight) Hydrogenotrpohic metabolism and 95% confidence intervals			Estimated cells/g (wet weight) Acetoclastic metabolism and 95% confidence intervals		
	MPN/g	CI – lower limit	CI – upper limit	MPN/g	CI – lower limit	CI – upper limit
GG	4.5	1.1	18	2	0.28	14
MBS	190,000	74,000	510,000	22,000,000	8,400,000	58,000,000
UBP	1,300	470	3,500	170	64	440

Despite potential inhibition of acetoclastic methanogenesis in the presence of hydrogen, acetate-amended culture medium showed high rates of methanogenesis in MBS and UBP samples. Acetate can promote growth of hydrogenotrophic methanogens, and bicarbonate in the growth medium could have allowed hydrogenotrophic methanogens to thrive even without amending these cultures with CO_2. Phylogenetic analyses support this theory.

DNA extractions from the two enrichment cultures from the MBS site yielded 73 clones. Ten different OTUs were identified at the 95% cut-off from these cultures, representing at least four major taxonomic groups (Supplemental Table; Appendix A). Most of the sequences (representing 93% of the total clones) belonged to the Methanomicrobiales (93-99% sequence identity) and were affiliated with three different, halotolerant genera (*Methanofollis*, *Methanospirillum, Methanocalculus*) as well as unclassified groups. Rarer OTUs were affiliated with an unclassified Archaea clone Tomm05_1274_3_Bac8 from a methane seep [FM179889] (81% sequence identity), an unclassified Euryarchaeota clone GLY2A01 [AB244743] (94% sequence identity) from a mesophilic anaerobic glycerol-fed digestor, and *Methanothermobacter* spp. (89% sequence identity). No Crenarchaeota were detected in the cultures.

Figure 2.2 clearly shows that the community composition from these cultures differs greatly from the community composition indicated by environmental sequences at the MBS site. This discrepancy may be due to the incubation temperature of the MPN bottles leading to changes in methanogenic community composition. *Methanomicrobiales,* which did not make up a large portion of environmental sequences

from the MBS site, dominated culture sequences. This is in contrast to environmental sequences from the lower-temperature UBP site, that are more similar to the cultures, suggesting that the low temperature incubation of the MPN cultures may have selected for methanogens within this order.

Inferences from archaeal diversity from environmental 16S rRNA gene sequences

Of the 490 16S rRNA gene sequence clones retrieved from the four ETGF sites (Appendix A), the environmental clone sequences ranged from 84-100% similar to sequences in GenBank, indicating the possibility of novel organisms. For instance, several sequences could be identified only as "unclassified Crenarchaeota" or "unclassified Euryarchaeota," as they grouped only with other environmental sequences that are distantly related to known Archaea, and could not be place confidently at the genus level (95% identification with 16S rRNA).

Table 2.4. Diversity statistics. The abundance-based coverage estimator (ACE) and Chao1 were estimated using EstimateS, version 8.2 (http://viceroy.eeb.uconn.edu/EstimateS). The evenness, Shannon-Wiener Index, and Simpson's Index were calculated using equations given by Hill et al. (2003).

Site	Mat type	# clones	Phylotypes observed	ACE	Chao1	Evenness (E)	Shannon-Wiener (H)	Simpon's Index (D)
GG total	Orange streamers	106	37	114.3	115.4	0.69	2.49	0.2
MBS total	Cyanobacterial mat	248	56	109.9	600.5	0.72	2.89	0.13
LTS	Cyanobacterial mat	35	15	19.8	21	0.47	1.27	0.08
UBP	Thick orange and pink mat	97	6	12	12	0.24	0.44	0.82

These unclassified microorganisms cannot be further described in terms of phenotypic traits, but genetic distance from other microorganisms may give clues as to the evolutionary pathway along which these organisms evolved.

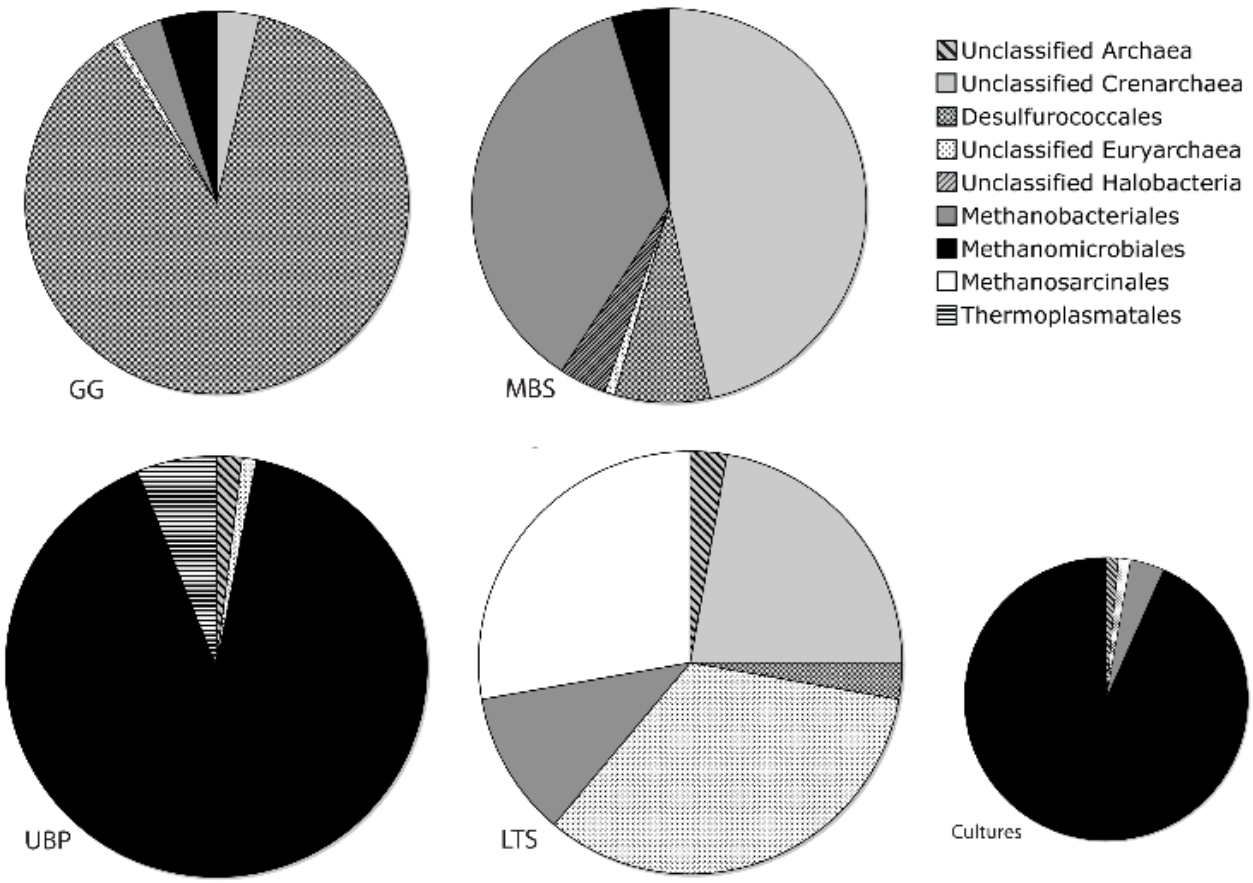
In addition to unclassified Archaea, six major taxonomic groups were retrieved (Supplemental Table; Appendix A). These groups are phylogenetically distributed predominantly within the phyla Crenarchaeota and Euryarchaeota. No Korarchaeota or Nanoarchaeota were identified, although sequences such as MBS-17 may be more closely related to these groups than Crenarchaeota (Figure 2.3). In many cases, ETGF archaeal sequences were phylogenetically more closely related to each other than to previously cultured relatives, despite differences in site chemistry or temperatures. An example of this is can be seen within the Thermoplasmatales in Figure 2.3.

Two OTUs were retrieved from three ETGF clone libraries and were also enriched in culture (Supplemental Table; Appendix A). The first OTU, representing ~19% of all ETGF clones, was affiliated with *Methanothermobacter thermautotrophicus* [NR_028241], order Methanobacteriales (95-99% sequence identity) (Figure 2.3). This hydrogenotrophic autotroph is the only recognized thermophile in the order (Kletzin, 2007). The second OTU, also representing ~19% of the clones in all libraries, was affiliated with a *Methanospirillum* spp. [AY454787] of the Methanomicrobiales. Two additional OTUs, affiliated with the *Thermoprotei* class in the Crenarchaeota, were found in three ETGF clone libraries but not the enrichment cultures. These two OTUs represented ~45% of all of the clones retrieved in this study and these clone sequences formed unique clades within the *Thermoprotei* (Figure 2.3). One of the OTUs, with 25%

of all ETGF clones and predominantly from the GG site, was affiliated with the fermenting, hyperthermophilic *Desulfurococcus* spp. (91-95% sequence identity) clone GBS_L3_B06 [DQ490017] from sediment in the 80˚C Great Boiling Spring in Nevada, USA. The temperature range for *Desulfurococcus* spp. growth is between 63 and 90°C, with optimum growth between 80 and 82˚C (Perevalova et al., 2005), which only half of the ETGF sites (GG and MBS) reached (Table 2.1). Phylogenetically, some of the ETGF clones clustered with *Aeropyrum pernix* (Figure 2.3), another genus of the order *Desulfurococcales* that is a strictly aerobic, hyperthermophilic heterotroph (Sako et al., 1996). The other OTU found in three ETGF clone libraries, with 21% of all ETGF clones and predominately from the MBS site, was affiliated (92-95% sequence identity) to the clone KOZ183 [EF156623] from the Norris Geyser Basin in Yellowstone National Park (Korf et al., 2007). Three OTUs, amounting to <2.5% of all ETGF clones, were found in two clone libraries. One of these OTUs was retrieved from the MBS and GG site and represented most of the enrichment cultures, being affiliated (93-99% sequence identity) to *Methanofollis liminatans* [nr_028254] (Appendix A; Figure 2.3).

The MBS site had remarkably high OTU (i.e. "species") richness according to Chao1 calculations, whereas the GG site had the highest based on ACE values (Table 2.4). Because of the high number of low-abundance OTUs, Chao1 is likely to represent diversity more accurately than ACE values (Hughes et al., 2001). Regardless, the UBP site had the lowest OTU richness according to both estimators. OTU evenness was greatest at the MBS site, and least at the UBP site. As the H' index includes rare OTUs in

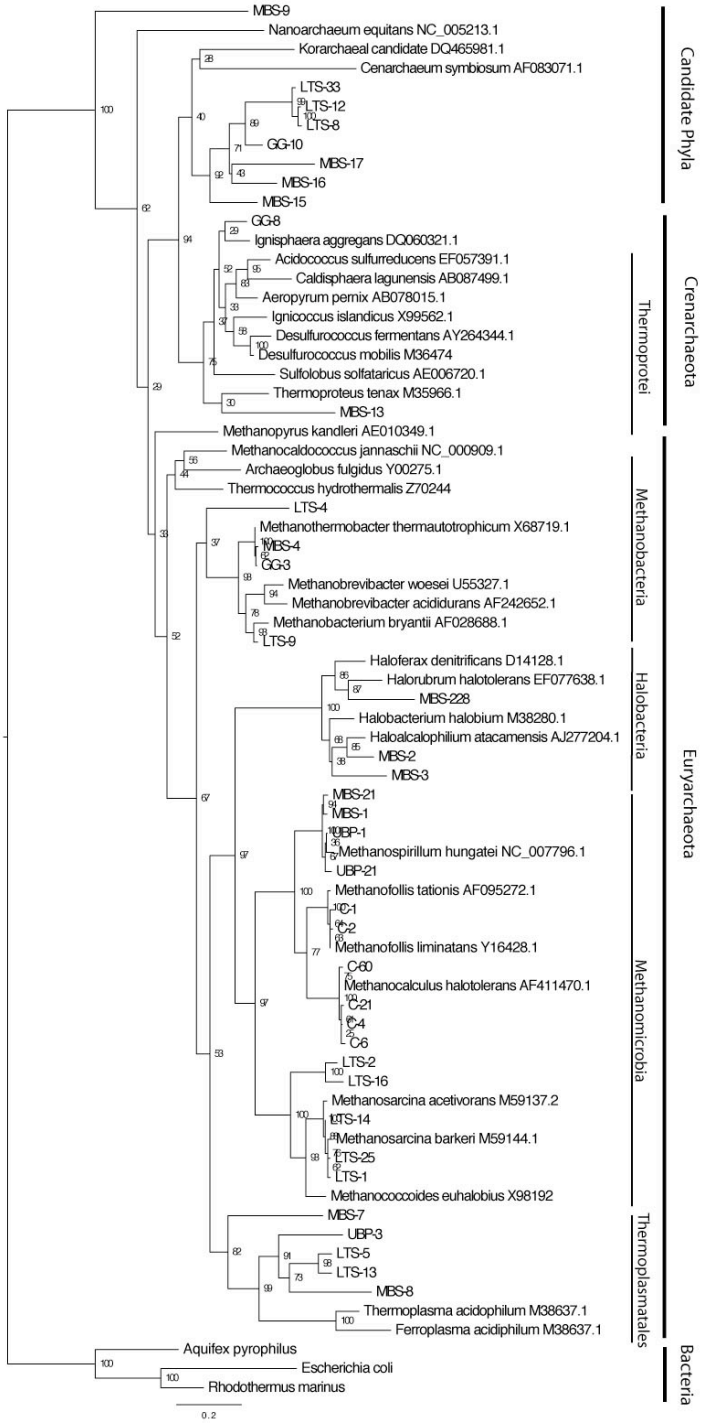
Figure 2.2: Phylogenetic Composition of Archael Community at 4 ETGF Sites, and within MPN Cultures.



determining diversity and the D index considers the most abundant OTU (Hill et al., 2003), this distinction was apparent for the ETGF clone libraries, in that the H' values suggested that most- to least-diverse sites were MBS > GG > LTS > UBP, while the D values ranked the sites as LTS > MBS > GG > UBP.

The higher OTU richness from MBS corresponded to higher diversity overall, especially because of the taxonomy of rarer OTUs (Supplemental Table). For example, three OTUs affiliated with the Halobacteria were only from MBS, even though they represented ~2% of all ETGF clones. These OTUs had 96-99% sequence identity to previously retrieved clones from a hypersaline desert [EU869367] and solar saltern [FN391291], and were phylogenetically related to previously described strains within the genera *Haloalcalophilium*, *Halobacterium*, and *Halorubrum* (Figure 2.3). Also from the MBS site, two OTUs formed a novel clade with four other OTUs from the LTS and UBP sites within the *Thermoplasmatales* (Supplemental Table; Appendix A, and Figure 2.3). Two clone sequences, MBS-15 and MBS-17, formed a clade with *Cenarchaeum symbiosum*, a member of the recently proposed archaeal phylum Thaumarchaeota (Brochier-Armanet et al., 2008). Only two thermophilic representatives (de la Torre et al., 2008; Hatzenpichler et al., 2008) are members of this phylum, which consists of aerobic ammonia oxidizing Archaea (Spang et al., 2010). If further phylogenetic analyses confirm this placement, these sequences could represent new thermophilic members of this proposed phylum.

Figure 2.3: Maximum likelihood phylogenetic tree. Select environmental and cultured ETGF samples and cultured Archaea from the greengenes database. Environmental sequences are labeled with site names; cultured samples are labeled as "C" instead of the site name.



RELEVANCE OF METHANOGENESIS AT EL TATIO GEYSER FIELD

In general, the diversity of Archaea from continental geothermal sites has been underrepresented, and consequently our foundational understanding of methanogenesis in these settings is poor. ETGF offers a unique opportunity to explore methanogen diversity, especially because of the exceptionally high As concentrations. The appearance of high concentrations of dissolved CH_4 at and below 65˚C in some features at ETGF suggests that hydrothermal activity alone is likely not responsible for CH_4 production, but rather that the CH_4 is microbially produced within the features.

Of the 16S rRNA gene sequence clone libraries resulting from the four ETGF sites, 5 of the 16 OTUs were represented by cultured isolates (Supplemental Table). The methanogenic communities were comprised of *Methanobacteriales* and *Methanomicrobiales*, represented at GG in approximately equal abundances, *Methanobacteriales* at MBS, and *Methanosarcinales* at the LTS site. *Methanomicrobiales* dominated at the UBP site. These results surpass the previous cultivation research that resulted in *M. tationis* (Zabel et al., 1984).

This is the first report of archaeal community diversity at El Tatio Geyser Field. Diversity estimates are high, especially at the MBS site. Phylogenetic resolution for some environmental 16S rRNA gene sequences from ETGF could not be resolved, including potentially novel thermophilic clades within each of these orders. Extremely high As concentrations are toxic to methanogens (Sierra-Alvarez et al., 2004), yet no correlation between methanogenic distribution and arsenic concentrations was observed

in this study. Our continuing work is evaluating the effect of both the high As as well as Sb concentrations on methanogen growth, as well as characterizing the previously uncultivated archaeal lineages at ETGF.

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Chapter 3: The effect of antimony on *M. thermautotrophicus* growth and arsenic resistance

ABSTRACT

The toxic metalloids arsenic (As) and antimony (Sb) are both found in extremely high concentrations at the El Tatio Geyser Field (ETGF), a high-altitude hydrothermal field in northern Chile. Because detoxification of both As(III) and Sb(III) involves the ArsB gene, and Sb(III) stimulates As(III) transport in *E. coli* (Meng et al. 2004), I hypothesized that the high Sb concentrations at ETGF increase microbial resistance to As.

Methanothermobacter thermautotrophicus, a hydrogenotrophic methanogen found in clone libraries from ETGF, was grown in the laboratory in media amended with Sb(III), As(III), and/or As(V) in concentrations within the range observed at ETGF; ~ 0.4-0.7 mM for As and ~0.01-~.02 mM for Sb. The optical density and daily methane production in cultures of *M. thermautotrophicus* were used to determine the affect of Sb(III), As(V), and As(III) on growth and metabolism.

Results for media amended with As(III) demonstrate complete inhibition of methanogenesis and growth, even in the presence of Sb(III). Amendment with As(V) allowed for inhibited growth and CH_4 production, followed by complete inhibition when As(V) was abiotically reduced to As(III). The presence of Sb(III) decreased As(V)

toxicity by altering abiotic processes controlling the fate of As; more total As was removed from solution, and no As(III) formed.

In cultures amended with Sb only, no inhibition of *M. thermautotrophicus* growth was observed. Daily CH_4 production over the first week of growth was higher in Sb-amended cultures than in control cultures, and peak production exceeded that of control cultures. Cultures grown in the presence of Sb were then used to inoculate new cultures to determine if previous exposure to Sb decreased the inhibitory affects of As. Those cultures showed the same inhibition of CH_4 production in the presence of As; however, results indicate that the concentration of Sb may be important in controlling the fate and toxicity of As.

INTRODUCTION

Arsenic (As) and antimony (Sb) are toxic metalloids often found in terrestrial hydrothermal environments, and both are found at extremely high concentrations in the discharge waters of the El Tatio Geyser Field (ETGF) of Chile. Their chemical similarities lead to the formation of comparable aqueous complexes and oxidative states, although the environmental fate of As and Sb are quite different. At ETGF, a significant fraction of the Sb partitions into the solid phase, associated with silica precipitation. A smaller fraction of As is removed from solution by adsorbing to iron oxides (Landrum et al., 2009).

The capacity of microorganisms to detoxify As and Sb is related, because the uptake and efflux mechanisms are the same in many Bacteria and Archaea (Meng et al., 2004). Although the ability of microorganisms to tolerate, and in some cases, respire As is well documented (Oremland, 2003; Stolz et al., 2006), the microbial detoxification and oxidation of Sb is not as well known due to the comparatively low natural abundances of Sb in the environment. However, the Sb concentration at ETGF is much higher than that typically encountered in natural systems, and serious consideration of the confounding impacts Sb may have on As toxicity (Gebel, 1998) need to be taken into account. For example, Sb concentrations at Yellowstone National Park range from 0.02-1.5 mM (Nordstrom et al., 2005), while at ETGF concentrations range from 8-21 mM. Arsenic concentrations are high at ETGF compared to other hydrothermal systems, with concentrations ranging from ~0.25-0.8 mM. In contrast, As concentrations range

between 0.001-0.05 mM at Yellowstone (Nordstrom et al., 2001), and at Mono Lake, the site of many arsenic metabolism studies, As concentrations are ~0.2 mM (Oremland et al., 2004).

ETGF is a unique hydrothermal field, located in northern Chile near the Chilean-Bolivian border (~22˚S latitude, 68˚W longitude), in the Andes mountains at an altitude of ~4200 m, and situated within the Atacama Desert ecoregion. This creates hyper-arid, high altitude environmental conditions unique to this geyser field. The UV-A and UV-B radiation at ETGF is ~3 times higher than in the central US, due to the altitude and low latitude. Hydrothermal waters exit geyser and spring features at ~86˚C, the boiling point at this altitude, and contain high concentrations of hydrothermal metals and metalloids.

The unique physicochemical conditions, including the high UV, reducing conditions, and hydrothermal setting are analogous to the environment in which early microbial communities lived (Hoehler et al., 2001; Phoenix et al., 2006). The high concentrations of As and other hydrothermal metal(loid)s may also be analogous to conditions within early ecosystems (Wolfe-Simon et al., 2009), and the ability of microorganisms to detoxify such metal(loid)s may have evolved quickly (Lebrun et al., 2003).

With a dearth of experimental evidence on Sb toxicity, the effects of As and Sb on microorganisms are considered to be roughly equivalent. Sb(III) is generally more toxic than As(III) (Lehr et al., 2007), and the trivalent species of both metalloids are more toxic than their pentavalent counterparts (Cervantes et al., 1994; de la Calle-Guntiñas et al., 1995). The uptake of As(III) and Sb(III) is a competitive process (Filella et al., 2007), so

that the presence of one species may affect the toxic effects of the other. However, the oxidation mechanisms for As(III) and Sb(III) are different (Lehr et al., 2007), so that microorganisms capable of tolerating As may not be capable of tolerating Sb, and vice versa. This may be due to the larger ionic radius and weaker Lewis acidity of Sb anions (Filella et al., 2007).

At ETGF, As(III) discharges from geyser and spring features, and is oxidized to As(V) along discharge transects. In addition to photooxidation and other abiotic processes, As is oxidized biotically by microbial mat communities, so that As(V) concentrations increase with distance from geyser sources, and increase more rapidly in the presence of mat communities (Landrum et al., 2009). Many of these communities are dominated by *Chloroflexi* (A.S. Engel et al., unpublished data), and contain diverse populations of chemoheterotrophic and chemolithotrophic Bacteria and Archaea.

The effect of high concentrations of metalloids such as selenium, arsenic and antimony on Archaea previously was studied in microcosms using consortia from anaerobic sludge reactors (Field et al., 2004). Although methanogens and some other microorganisms detoxify by methylating arsenic (Bentley & Chasteen, 2002), the most well-studied detoxification mechanism is the ArsC system, in which As(V) is reduced within the cytoplasm and expelled using an ATP-dependent transporter (Oremland, 2003). ArsB, an arsenite efflux membrane protein that operates independently of ATP, is also well-characterized. ArsC, ArsB, and other cell machinery required for As detoxification was found in both Bacteria and Archaea (Lebrun et al., 2003; Lin et al., 2006; Stolz et al., 2006). Arsenate respiration has been discovered in Crenarchaeota

(Huber et al., 2000), and arsenite oxidases were found in Crenarchaeota and several Euryarchaeota species (Stolz et al., 2006).

Interest in As- and Sb- resistant microorganisms developed in recent years due to the role microorganisms play in determining groundwater chemistry, redox, and the subsequent transport behavior of toxic metals. Although researchers previously investigated the toxicity of arsenic on methanogenic Archaea (e.g., Field et al., 2004; Sierra-Alvarez et al., 2004), most focus on the capacity of methanogenic consortia or model species to produce volatile methylated metalloids (e.g., Bentley & Chasteen, 2002; Meyer et al., 2008; Michalke et al., 2000). No studies of As toxicity on methanogens included Sb, and no previous studies were done testing the toxicity of Sb on methanogens, although the ability of methanogens to produce methylated Sb in high Sb conditions was observed by Michalke et al. (2000).

I focused on the affects Sb and As have on methanogenic population growth and methane production in a model organism. An isolate of *M. thermautotrophicus* was grown in media amended with As(III), As(V), Sb(III), or a combination of As and Sb(III) in concentrations that approximate those found in the field.

METHODS

Culture medium of *Methanothermobacter thermautotrophicus*

Because an environmental isolate from ETGF could not be obtained with sufficient biomass and growth rates for experimentation, a isolate of

Methanothermobacter thermautotrophicus (ATCC #29183) was inoculated anaerobically and aseptically into the maintenance medium described by Zeikus and Wolfe (1972).

Briefly, the medium contains 83.5 parts distilled water, 5 parts 8% Na_2CO_3, 2.5 parts mineral solution 1 (6g/L K_2, 5 parts mineral solution 2 (, 2 parts cysteine-sulfide reducing agent, and 1 part each Wolfe's vitamin solution and Wolfe's trace-metal solution). Resazurin was added as an anaerobic indicator, and the pH of the solution was adjusted to 7.2 by the addition of 6 N HCl. The solution was pre-reduced and anaerobically sterilized according to the protocol provided by Holdeman and Moore (1972). The vitamin and trace-metal solutions were filter-sterilized and added to individual tubes after autoclaving.

Cultures were inoculated in a Coy anaerobic chamber with an atmosphere of 1.5% H_2 in N_2 and CO_2 (Coy Laboratory Products Inc., Grass Lake MI, USA). Hungate tubes containing 10 mL of media was inoculated with 1 mL of *M. thermautotrophicus* from the same stock culture. Each bottle had a 140kPa overpressure of 80:20 H_2:CO_2. Cultures were vacuumed and re-gassed daily, and incubated anaerobically at 65˚C during growth.

Culture amendments

Cultures were amended with either As(V), As(III), Sb(III), or a combination of Sb(III) and an As species. Two different concentrations within the range observed at ETGF were used for each amendment (Table 3.1). Experiments were done in triplicate, and a sterile control was included for each amendment at each concentration. Triplicate

cultures grown without As or Sb amendments were grown under the same conditions, including amendment with acetic acid, as controls.

Arsenic was added either as filter sterilized sodium arsenite ($NaAsO_2$) or potassium arsenate (KH_2AsO_4). Culture tubes were inoculated to final concentrations of 0.4 mM or 0.67 mM total As.

Antimony was added as filter-sterilized antimony acetate ($Sb(H_3COO)_3$), to a final concentration of 12 mM or 21 mM. Because antimony acetate dissociates to antimony trioxide and acetic acid in water, and acetate can stimulate CO_2 uptake in hydrogenotrophic methanogens, filter-sterilized 2 mM acetic acid stock solution was added in appropriate volumes to each culture to a final concentration of 0.075 mM acetic acid.

Additionally, several sets of cultures were amended with both As and Sb. In these mixed-amendment cultures, either As(III) or As(V) was added at a 0.4 mM concentration. Antimony was added to final concentration of 12 mM, and acetic acid added to a final concentration of 0.075 mM.

Pre-exposure experiments

Because previous authors showed increased microbial growth in Sb-amended media when communities were pre-exposed to Sb (Lehr et al., 2007), *M. thermautotrophicus* grown in Sb-amended media were then exposed to As(III) and Sb(III) to test whether resistance to As(III) improved. Resistance was tested using two

Table 3.1: Culture amendments and final concentrations for (a) toxicity experiments, and (b) pre-exposure toxicity experiments. Triplicate cultures and one sterile control were completed for each amendment.

(a)	$NaAsO_2$	KH_2AsO_4	$Sb(H_3COO)_3$	$CH_3(COOH)$
Control	0	0	0	0.075 mM
As(III), low	0.4 mM	0	0	0.075 mM
As(III), high	0.67 mM	0	0	0.075 mM
As(V), low	0	0.4 mM	0	0.075 mM
As(V), high	0	0.67 mM	0	0.075 mM
Sb(III), low	0	0	12 μM	0.075 mM
Sb(III), high	0	0	21 μM	0.075 mM
As(III) Sb(III)	0.4 mM	0	12 μM	0.075 mM
As(V) Sb(III)	0	0.4 mM	12 μM	0.075 mM

(b)	$NaAsO_2$	KH_2AsO_4	$Sb(H_3COO)_3$	$CH_3(COOH)$
High Sb(III) As(III)	0.4 mM	0	21 μM	0.075 mM
Low Sb(III) As(III)	0.4 mM	0	12 μM	0.075 mM
High Sb(III) As(V)	0	0.4 mM	21 μM	0.075 mM
High Sb(III) As(V)	0	0.4 mM	12 μM	0.075 mM

different concentrations of Sb(III), and cultures with As(V) in place of As(III) were also conducted.

In these experiments, *M. thermautotrophicus* was grown in media with a final concentration of either 12 mM or 21 mM Sb(III) for 4 weeks. After 4 weeks, 1 mL aliquots of stock cultures were added to 10 mL cultures with the same concentration of

Sb(III). Cultures were amended with either 0.4 mM As(III) or 0.4 mM As(V), and with CH_4COOH to a final concentration of 0.075 mM (Table 3.1). Experiments were done in triplicate, with one sterile control for each set of parameters, and one set of control cultures. Control cultures were inoculated with 0.5 mL of cells from the 12 mM stock and 0.5 mL of 21 mM Sb(III) stock, although no Sb or As was added after inoculation.

Optical density

Growth was measured turbidimetrically at 660 nm in matched test tubes with a LaMotte Smartspectro (LaMotte Company, Chestertown MD, USA) spectrophotometer. Sterile culture medium was used to blank the spectrophotometer. Results from triplicate cultures were averaged.

Gas Chromatography

Daily methane production was determined via a SRI 8610C gas chromatograph (SRI Instruments, Torrance CA, USA) using a flame ionization detector and a Restek column (Restek, Bellefonte PA, USA). Samples were run at 35°C and an injection size of 100 mL. The concentration of CH_4 in the media was calculated using Henry's law, and a Henry's law constant of 1.5×10^{-3} $M \cdot atm^{-1}$ (Hine & Mookerjee, 1975). Calculations were corrected for the overpressure inside culture tubes, which were kept at 1.43 atm.

Arsenic Speciation and total arsenic concentration

Arsenic speciation data were collected by HPLC using a Waters 484 UV detector at 208 nm (Waters, Milford MA, USA). A 0.1% H_2SO_4 eluent was used at .7 ml/min, and sample injections were 50 μL in volume.

Inductively coupled plasma mass spectrometry was performed using an Agilent 7500ce Quadrupole ICP-MS to determine final concentration of As in 4 samples; a 0.67 mM As(V)-amended sterile control and culture, and a 0.4 mM As(V)- 0.012 mM Sb(III)-amended sterile control and culture. Samples were filter-sterilized prior to acidification and analysis.

Mineral Precipitate

Environmental scanning electron microscopy (ESEM, FEI XL30) with energy dispersive X-ray spectroscopy (EDAX) was used to image and qualitatively analyze elemental composition of precipitates. Culture medium was filtered with a 0.2mm carbon filter to catch precipitates, which were washed with DI water to remove salts.

RESULTS

Methane production

Control cultures increased in daily methane production until reaching a peak of ~22 mmoles/day after 10 days of incubation (Figure 3.1). Production of CH_4 declined slowly, dropping to ~18 mmoles/day at 60 days, and declining more rapidly after that

date. Rates of growth and decline varied between samples, leading to a higher deviation between cultures at the beginning and end of the experiment (see Supplementary Table, Appendix B). Sterile control cultures for Sb(III), As(V), As(III), and mixed cultures showed no significant methane production.

Cultures amended with only As(III) did not produce methane at any time during the experiment (Figure 3.1a). The addition of Sb(III) to As(III) cultures did not change the results, and no growth was observed.

In cultures amended only with As(V), results were dependant on As(V) concentration (Figure 3.1b). At the highest concentration tested, 0.67 mM, methane was produced in low concentrations for the first few days of incubation, but CH_4 production ceased after the third day. At 0.4 mM concentrations of As(V), methane production was significantly inhibited in comparison to control cultures after day 5 of growth, and ceased within one month of incubation.

Cultures amended with a mixture of As(V) and Sb followed the same growth curve as the As(V)-only cultures; however, CH_4 production continued over the 60-day sampling period. Inhibition of CH_4 production varied widely in these samples, with some samples showing little to no inhibition, and others exhibiting complete inhibition at the end of 2 weeks (Appendix B). Because production curves are averages of the triplicate cultures, the observed decline in daily CH_4 production after ~15 days in the Sb(III) and As(V)-amended culture is due to the inhibition of one of the three replicates.

In cultures amended with Sb, CH_4 production exceeded that of control cultures by up to 30% (Figure 3.1c). Methane production peaked during the third week of

incubation, at which point methane production dropped slowly and appeared to stabilize. The amount of methane produced did not appear to correlate closely with the concentration of Sb in solution, although the higher-Sb media consistently produced more methane than either the low-Sb media or the control cultures after one month. Over time, methane production by Sb-amended cultures decreased to approximately half the peak concentration, but continued to produce methane at a concentration comparable to the control culture even after 60 days.

Pre-exposure experiments

Control cultures showed a decrease in methane production when cells were pre-exposed to Sb, with peak CH_4 production ~18 μmoles/day (Figure 3.2). Control cultures died off after ~2 weeks of incubation, dropping to ~10 μmoles/day CH_4, but rebounded before the end of the experiment.

Pre-exposed *M. thermautotrophicus* do not show increased resistance to As(III), and complete inhibition of cells was observed through the duration of the experiment. The concentration of Sb does not affect As(III) inhibition.

Methane production in As(V)-amended cultures showed inhibition dependent on Sb concentration. Cultures amended with high concentrations of Sb did not exceed 2 μmoles/day CH_4. As(V)-amended cultures with low concentrations of Sb showed initially rapid CH_4 production, followed by a rapid decline. Complete inhibition does not occur in this culture, however, and CH_4 production rebounds after ~2 weeks of incubation.

Figure 3.1a: Daily methane production, in μmoles, for (a) As(III)-amended cultures. Points shown are average values of triplicate cultures; SC is the standard control culture. Standard deviations are shown in Appendix B. Low As= 0.4 mM, high As= 0.67mM.

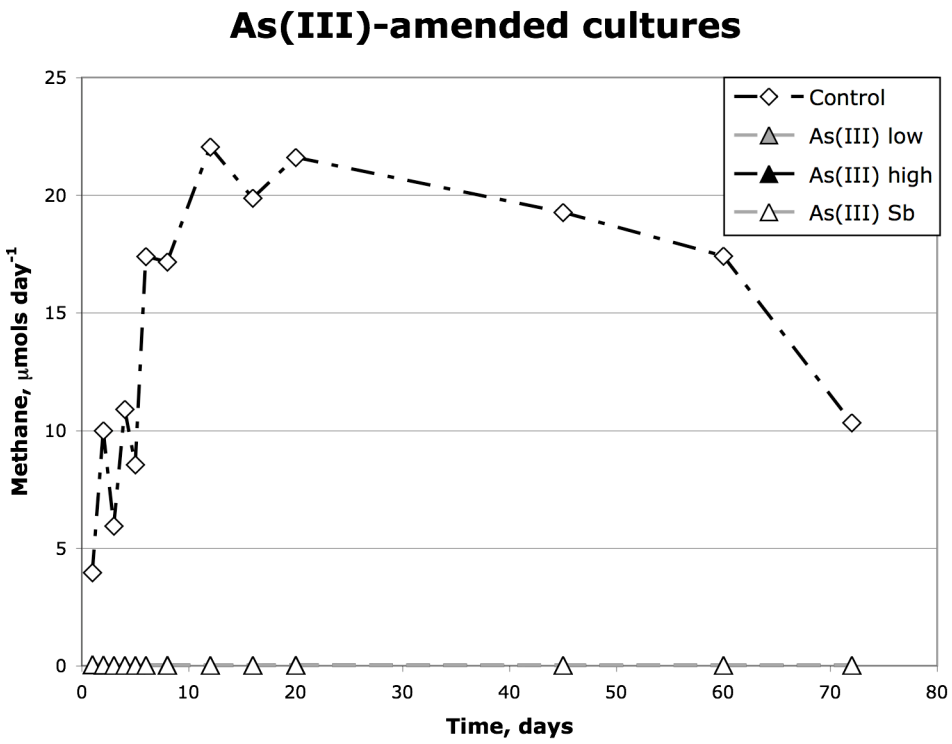


Figure 3.1b: Daily methane production, in μmoles for (b) As(V)-amended cultures. Points shown are average values of triplicate cultures; SC is the standard control culture. Standard deviations are shown in Appendix B. Low As= 0.4 mM, high As= 0.67mM.

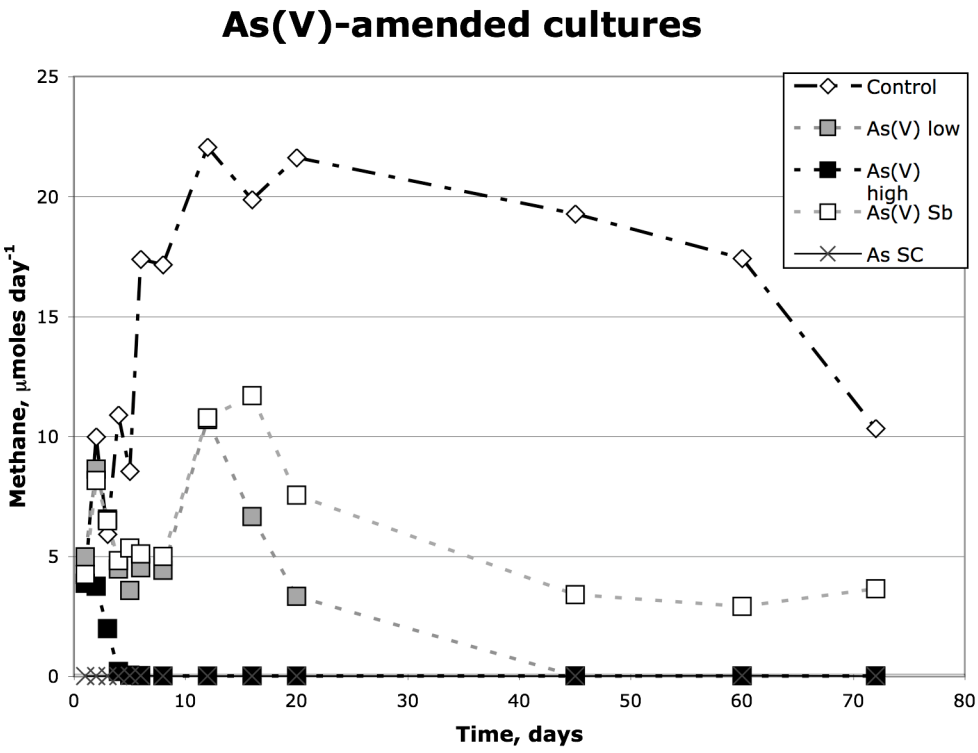
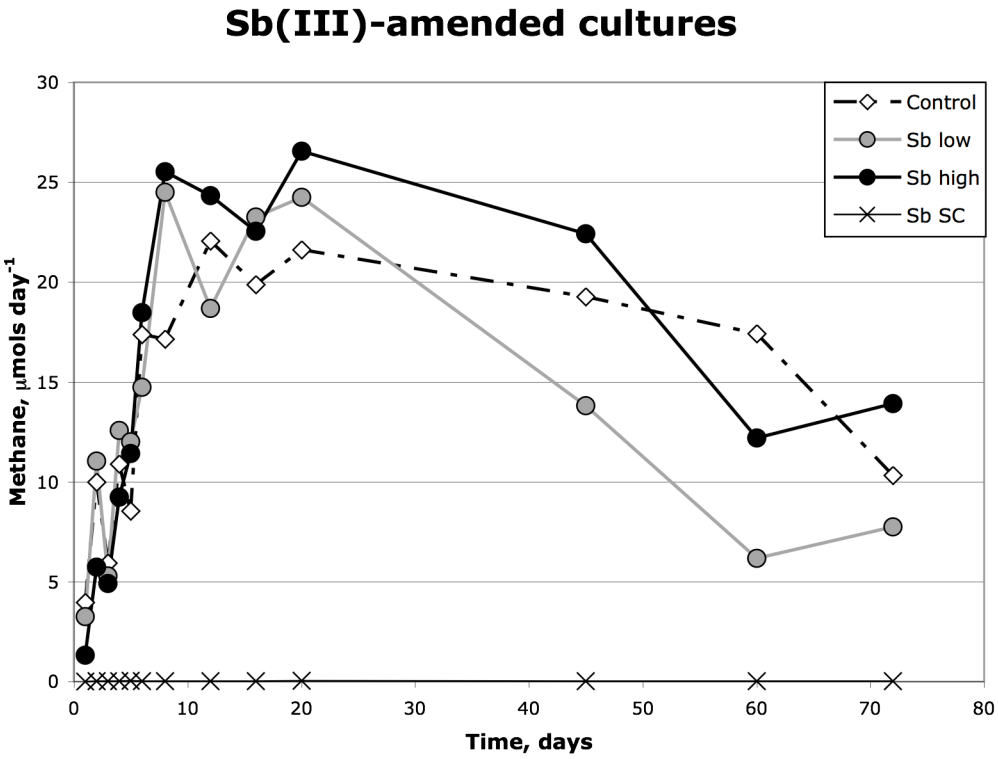


Figure 3.1c: Daily methane production, in μmoles, for (c) Sb(III)-amended cultures. Points shown are average values of triplicate cultures; SC is the standard control culture. Standard deviations are shown in Appendix B. Low Sb= 12 μM, high Sb= 21 μM.



This is followed by another decline in production, followed by complete inhibition after ~22 days.

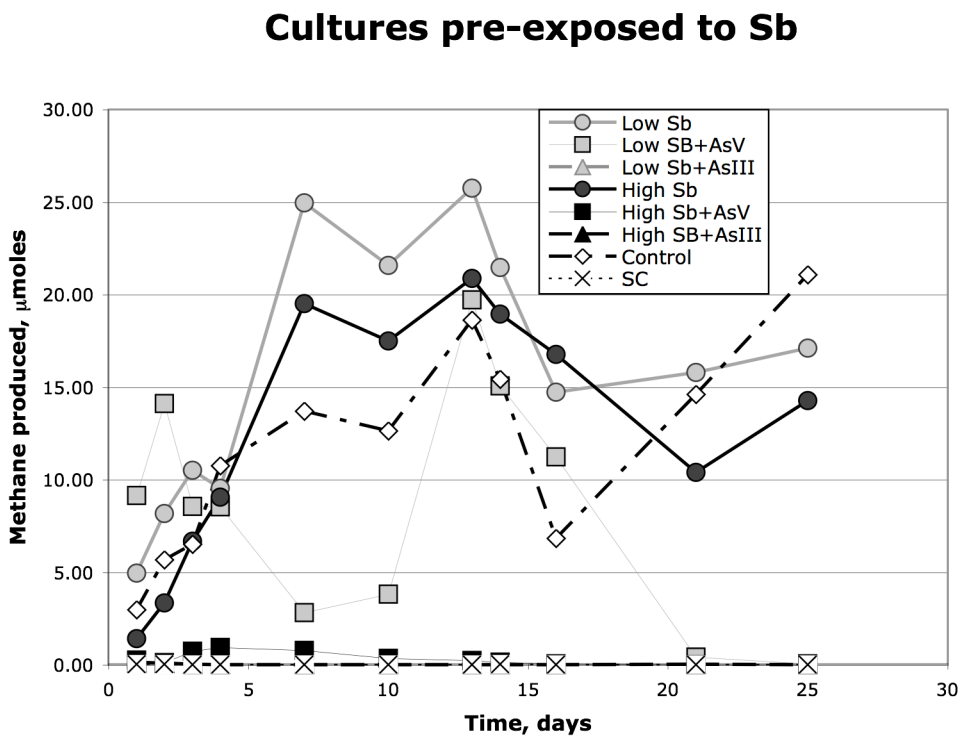
Sb-amended cultures peaked ~2 weeks into the experiment, and exceeded the control cultures in methane production until the end of the third week. In this set of experiments, the low-Sb concentration cultures produced more methane than the high concentration cultures. The increase in CH_4 production is approximately equal for both Sb-amended cultures and the control cultures for the first four days of incubation; after which time the rate of control culture production decreases.

Arsenic speciation

Arsenic speciation shows a decrease in total As over time, as well as the production of As(III) in As(V)-amended cultures (Figures 3.3 and 3.4). As(III)-amended cultures show trace amounts of As(V) (less than 0.1 mM), and a small decrease in the total amount of As. Arsenic speciation was only measured over the first five days of incubation for As(III)-amended cultures.

The As(V)-amended cultures show a decrease in As(V) concentration over time. By the 2nd day of incubation, the As(V) in high concentration As(V) samples decreased by half in both inoculated and sterile media. Both the low concentration As(V) media and the combined As(V)-Sb(III) media show a similar pattern. Arsenate decreases rapidly, and is less than half its original concentration in both inoculated and sterile media by day 3. By day 8 all cultures have less than 0.1 mM As(V), and by day 40 only trace amounts of As(V) are present in cultures (Figure 3.3).

Figure 3.2: Daily methane production of all cultures in pre-exposure experiments, in μmoles. Low Sb= 12 mM, high Sb= 21 mM. Arsenic concentration was 0.4 in As-amended cultures.



As(V)-amended cultures show an increase in As(III) starting at day 2 at both high and low concentrations (Figure 3.4). In inoculated cultures, the increase in As(III) is followed by a gradual decrease, so that by day 40 As(III) levels were 75% lower than at their peak for the high concentration cultures. The gradual decrease in As(III) is not as apparent in the low concentration As(V) culture. Sterile controls for both As(V) cultures also show formation of As(III) by day 2 of incubation, but As(III) concentrations are stable over time.

The presence of Sb(III) in As-amended cultures appeared to alter arsenic speciation. If both As(V) and Sb(III) were added to culture media, no As(III) formation occurs in either the inoculated culture or the sterile control.

Optical Density

Optical density measurements were done in conjunction with As speciation and CH_4 production measurements. In general, optical density was low for all samples, and did not exceed 0.045 for any of the cultures over a 60-day period (Figure 3.5).

As(III) cultures never exceeded an absorbance of 0.01 (Figure 3.5a). Sterile control cultures for all media showed the same result as As(III) cultures (data not shown).

As(V) cultures varied based on concentration, as well as amendment with Sb(III). The higher concentration As(V) culture initially had a higher absorbance, but by the twentieth day it was declining rapidly. The low As(V) concentration peaked in absorbance on day 23 day, but then declined at the same rate as the higher concentration (Figure 3.5b).

Figure 3.3: As(V) concentration over time for four different culture amendments. Average values for triplicate inoculated cultures and corresponding sterile controls (SC) are shown for an 0.67 mM As(III), 0.67 mM As(V), 0.4 mM As(V), and 0.4 mM As(V) with 0.021 mM Sb(III).

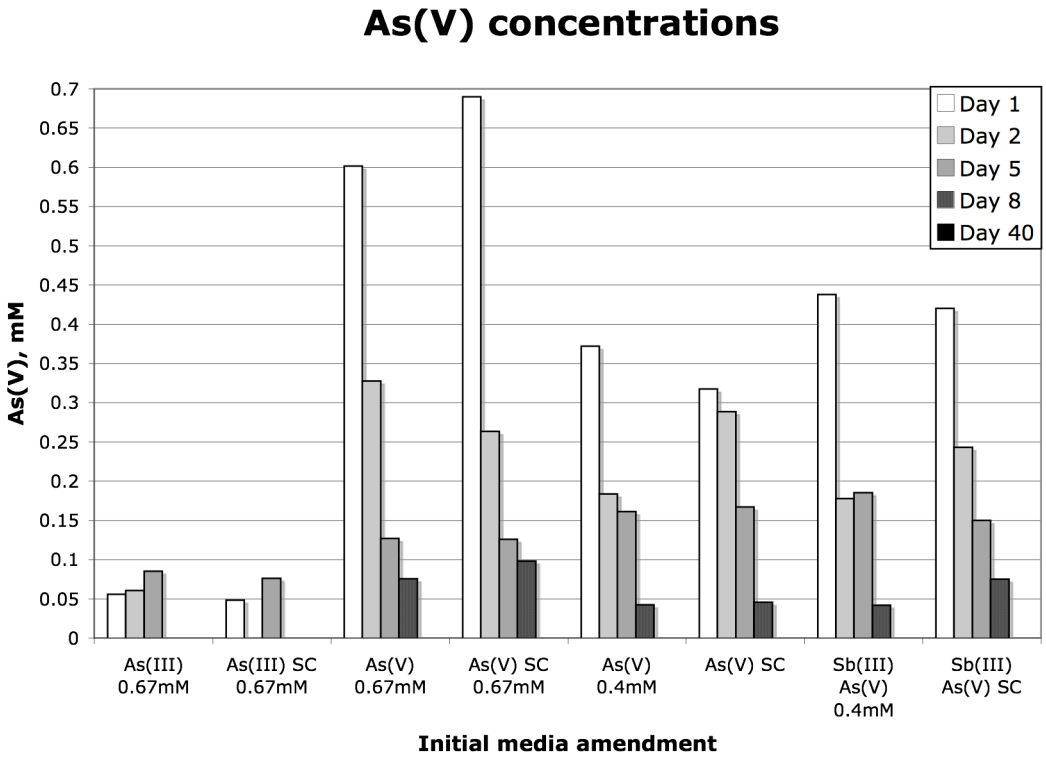
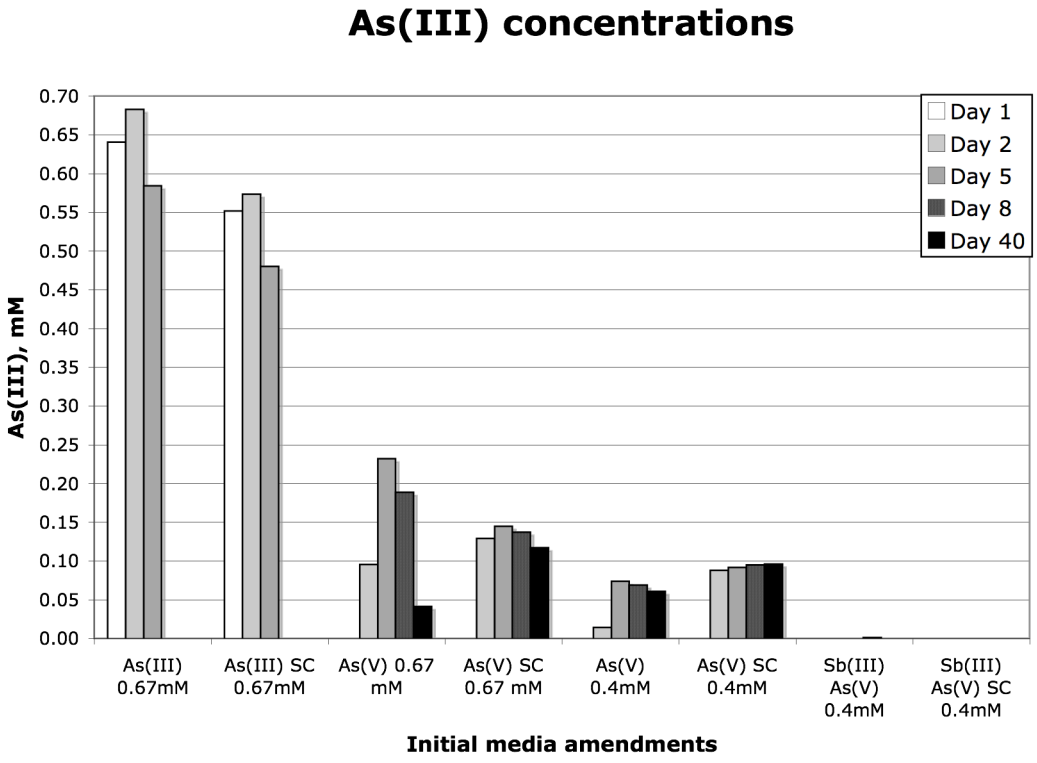


Figure 3.4: As(III) concentration over time for four different culture amendments. Average values for triplicate inoculated cultures and corresponding sterile controls (SC) are shown for an 0.67 mM As(III), 0.67 mM As(V), 0.4 mM As(V), and 0.4 mM As(V) with 0.021.



The As(V) culture also amended with Sb(III) followed the same growth pattern as the low concentration As(V) culture, increasing steadily until day 23 day, at which time it decreased slightly and began to plateau between 0.01 and 0.015.

Sb(III)-only cultures had higher absorbancy than As(V) or As(III) cultures, and were comparable to the control cultures (Figure 3.5c). The low-concentration Sb(III) culture plateaued around ~0.02. The high-concentration Sb(III) culture rose rapidly in absorbance, peaking at about two weeks and then declining to plateau at ~0.03, the highest OD. The control cultures were between the low- and high-concentration Sb(III) cultures, leveling off just under 0.03.

The optical densities of cultures in the pre-exposure experiments never rise above 0.03 (Figure 3.6). The peak for all cultures occurs around day 2 of incubation, followed by a decline in optical density. The only exceptions to this are the cultures amended with As(III), which never peaked, but maintained optical densities <0.01 throughout the experiment.

Neither of the cultures amended with As(V) showed significant growth; both reach a plateau ~0.01 absorbance units. The cultures amended with a low concentration of Sb showed the most rapid growth, and exceeded all other cultures. The high-Sb culture showed optical density results similar to the control cultures for the first 20 days of incubation. After 20 days, the rate of growth of the control culture increased, until it was about 0.02 absorbance units at the end of the experiment.

Figure 3.5a: Optical density for (a) As(III)-amended media. As low= 0.4 mM, As high= 0.67 mM.

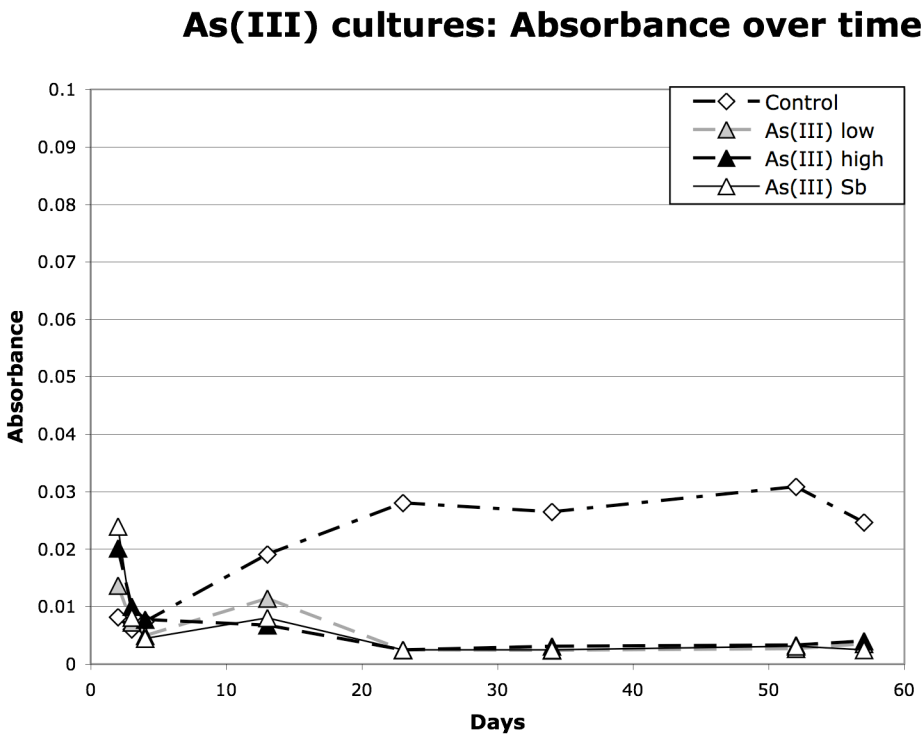


Figure 3.5b: Optical density for (b) As(V)-amended media. As low= 0.4 mM, As high= 0.67 mM.

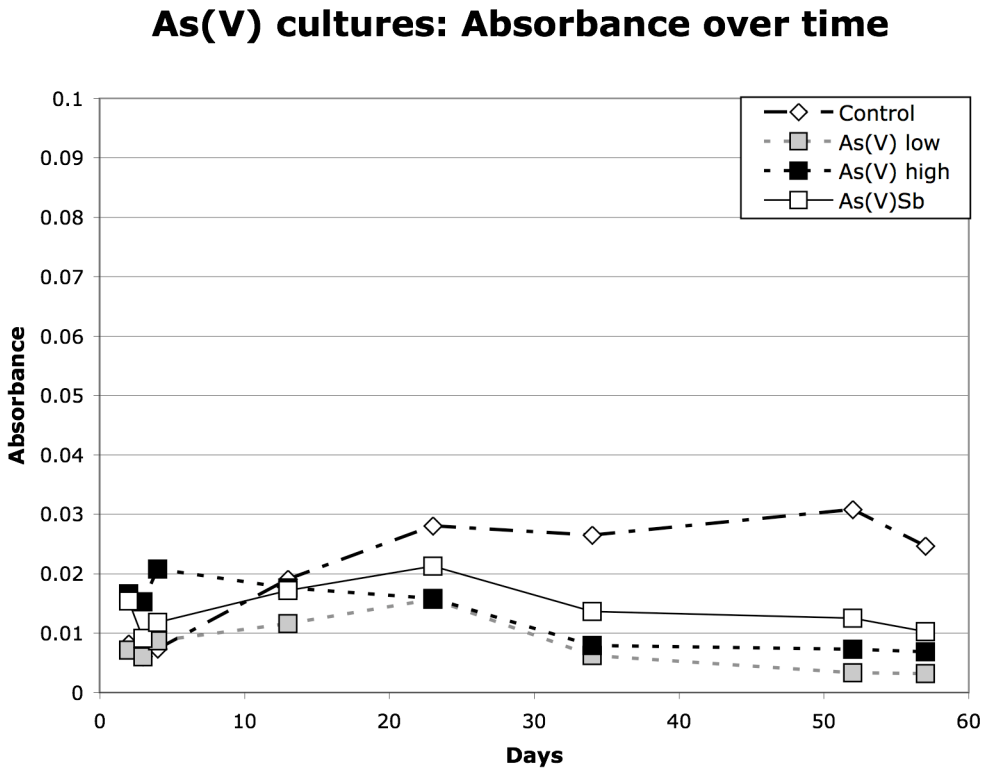


Figure 3.5c: Optical density for (c) Sb(III)-amended media. As low= 0.4 mM, As high= 0.67 mM.

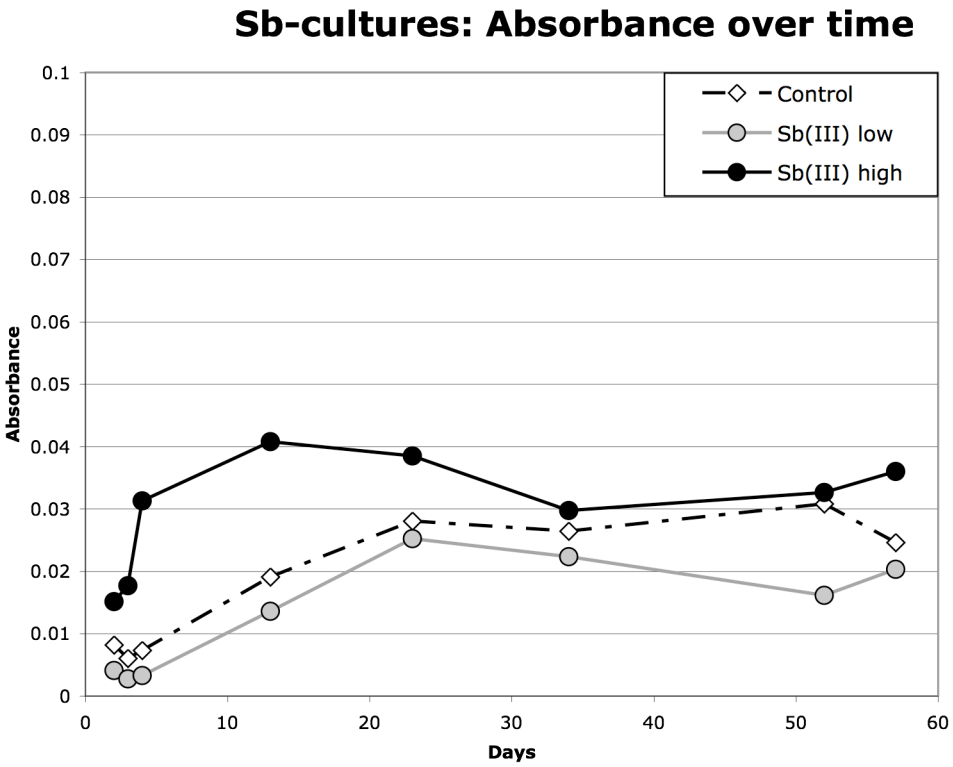
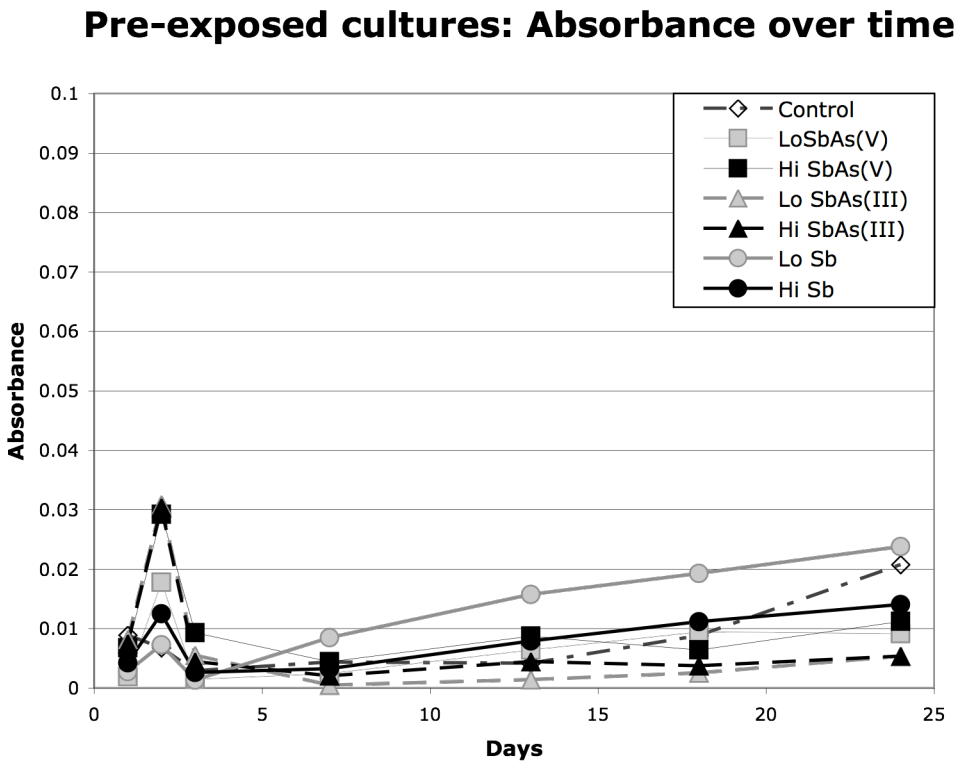


Figure 3.6 Optical density for Sb pre-exposure experiments. Low Sb= 12 μM, high Sb= 21 μM. Arsenic-amended cultures had final concentrations of 0.4 mM As.



Dissolved metals and mineral precipitates

Final As concentration for the 0.67 mM As(V)-amended cultures show that only ~0.1 mM total As was lost from the aqueous phase (Table 3.2). This is in contrast to HPLC data, which shows a net loss of ~0.5 mM in combined As(III) and As(V). Results are the same for both the inoculated and sterile control cultures.

Cultures amended with both As(V) and Sb(III) have final As_T concentrations of just over 0.1 mM. The sterile control culture has a concentration 0.04 mM higher than the inoculated culture. Combined As(III) and As(V) concentrations, as determined by HPLC, are slightly lower but also show higher As_T in the sterile control culture than in the inoculated culture.

Table 3.2: Total aqueous As concentrations after 60 days in inoculated cultures and sterile controls (SC). Initial concentrations were not measured using ICP-MS. Final As and Sb concentrations were taken at ~60 days.

	Initial As_T, mM	Final As_T mM	Initial Sb_T µM	Final Sb_T µM
As(V) culture	0.67	0.57	-	-
As(V) SC	0.67	0.56	-	-
As(V) Sb(III) culture	0.4	0.11	12	0.3
As(V) Sb(III) SC	0.4	0.16	12	0.1

A net loss in total Sb occurs in both inoculated and sterile control media. Although only a small fraction of the original Sb was present, the inoculated culture had 3x as much Sb than the sterile control after 60 days.

All cultures, including sterile controls, and control cultures with no As or Sb, formed grey and black precipitates during experimentation. SEM-EDAX analysis of

control cultures indicates those precipitates are iron sulfide.

In addition to the grey-black precipitates, yellow-orange precipitates were present in As(V)-amended cultures. SEM-EDAX of those precipitates indicated arsenic sulfide solids were present. The same precipitates were observed in cultures containing both As and Sb. SEM-EDAX analysis indicated that arsenic sulfides were present in those cultures, as well. Although some antimony was found in the solid phase as well, it was not associated with sulfur. The only element identified in conjunction with Sb was carbon.

DISCUSSION

As-amended cultures

As(III) completely inhibited *M. thermautotrophicus* growth and CH_4 production. Complete inhibition was observed at both high and low concentrations of As(III), and in cultures amended with both As(III) and Sb(III). A change in As(III) toxicity in the presence of Sb(III) was expected, but not observed. As(III) toxicity was not altered by pre-exposure of cells to Sb(III).

My data do not support the hypothesis that the uptake of As(III) and Sb(III) are competitive processes in *M. thermautotrophicus* at the concentrations observed at ETGF. Speciation and CH_4 production data indicate that low concentrations of As(III), ~0.10 mM, can completely inhibit growth of *M. thermautotrophicus* and methane production.

As(V) also inhibited growth of *M. thermautotrophicus* and methane production;

however, the gradual reduction of As(V) to As(III) appears to be the principal cause of toxicity. Speciation data suggest this reduction is an abiotic process, as results are the same for both biotic and abiotic cultures. Decreased production of CH_4 appears to correlate to the concentration of As(III) in solution. In high- concentration As(V) media, As(III) was ~0.10 mM by the second day of incubation, and CH_4 production, initially ~4 μmoles/day, was completely inhibited by the fifth day of incubation. In the low-concentration As(V) media, As(III) concentration exceeded 0.05 mM by the fifth day of incubation, but never reached 0.10 mM. Complete inhibition occurred between day 20 and day 40 day of incubation without further increases in As(III), and with an overall decrease in As concentration. These data suggest that even 0.05 mM As(III) is toxic over time.

The decrease in As_T observed over time in HPLC data for both inoculated and sterile control cultures. Although ESEM-EDAX shows some arsenic sulfide precipitates are present, ICP-MS results suggest that reduction of As(V) to As(III) does not account for all aqueous arsenic species. The formation of organoarsenical compounds in As-amended cultures could explain the discrepancy in HPLC and ICP-MS results, because only concentrations of As(III) and As(V) were determined via HPLC.

While the As_T is equivalent in sterile control and inoculated cultures at the end of the experiment (Table 3.2), HPLC data show inoculated cultures decrease in As(III) over time after initial formation of As(III) in As(V) media, while As(III) concentration remains fairly constant in sterile cultures (Figure 3.4). Although this could be due to the formation of arsenic sulfide precipitates, As_T concentrations suggest that the amount of

precipitate formed in inoculated and sterile cultures is equivalent. This may be indicative of a biological transformation of As(III) at low concentrations.

Sb-amended cultures

My results indicate Sb(III) has no negative effects on *M. thermautotrophicus* methane production. The initial rate of increase in daily methane production, as well as the peak methane production, exceeded the control cultures in both the initial and pre-exposure experiments. Growth of *M. thermautotrophicus*, as measured by optical density, suggests that increased microbial biomass is responsible for the increase in CH_4 production. This is contrary to what I hypothesized, because in most cases the toxicity of Sb(III) exceeds that of As(III).

After two months, almost all Sb was removed from both inoculated and sterile culture tubes. While SEM-EDAX did identify Sb in the solid phase, it was not associated with As or S. Carbon was associated with Sb precipitates, which could indicate adsorption of Sb onto *M. thermautotrophicus* cells. However, the removal of Sb from the sterile control culture suggests that precipitation is independent of the presence of cells. These results suggest the formation of Sb precipitate decreased the aqueous concentration of Sb, thus rendering it non-toxic to *M. thermautotrophicus*. Analyses of Sb concentration were not performed until the end of the experiment, and, thus, a timeline for the removal of aqueous Sb is not available.

Arsenic speciation data show that the removal of As from solution occurred gradually, and CH_4 production data suggest the gradual removal of Sb as well. In both

the initial and the pre-exposure experiments, CH_4 production increased at a faster rate in Sb-amended cultures than control cultures. Production peaked at approximately two weeks of incubation for control cultures, and after only ~1 week for Sb-amended cultures. Optical density data also suggest biomass of *M. thermautotrophicus* increases in the presence of Sb; however, biomass accumulation was low, and the difference observed is only ~0.01 absorbance units.

The control cultures did not produce as much CH_4 in the pre-exposure experiments as in the initial experiments. Although no Sb was added to the culture medium for the controls, the stock culture used to inoculate the controls contained Sb. The depressed CH_4 production relative to previous controls suggests that exposure to Sb, followed by Sb removal, decreases CH_4 production. Additionally, the decline and then dramatic increase in CH_4 production in control cultures after ~16 days may be indicative of a population shift in which Sb-exposed *M. thermautotrophicus* is dying off, leaving a population that was never exposed to Sb.

Sb(III) and As(V) amended cultures

The presence of Sb(III) in culture media also appeared to alter the toxicity of As(V), most likely by preventing the reduction of As(V) to As(III). The presence of Sb(III) in As(V)-amended cultures appeared to affect both the amount of CH_4 produced, as well as the amount of time that cultures of *M. thermautotrophicus* could survive.

In cultures amended with both As(V) and Sb(III), the net loss of As(V) from the system did not result in the formation of As(III) (Figure 3.3 and 3.4). ICP-MS data also

show a significant decrease in As_T concentration. This removal of As_T from cultures is not observed in As(V)-only amended cultures, and suggests that the presence of Sb(III) alters the geochemistry of the system to favor removal of As from the aqueous phase. More As_T is removed from inoculated cultures than from sterile cultures, which could suggest an increase in arsenic sulfide precipitation, or the formation of methylated arsenic gases, stimulated by the presence of Sb.

As(V)-Sb-amended cultures grew at a similar rate to cultures amended with As(V) only for the first ten days of incubation, at which time the As(V) cultures peaked at ~10 μmolesCH_4/day. The As(V)-Sb cultures continued to produce CH_4, peaking at ~12 μmolesCH_4/day, and then reaching a plateau ~3 μmolesCH_4/day. Speciation data suggest a large proportion of the As_T at the end of the experiment is As(V). This suggests that low levels of As(V), ~0.10 mM, inhibits *M. thermautotrophicus* growth and CH_4 production over extended time periods.

Pre-exposure of *M. thermautotrophicus* to Sb also affected As(V) toxicity. When cultures contained high concentrations of Sb, CH_4 production by *M. thermautotrophicus* was more inhibited than with As(V) alone. Because all cultures amended with a mixture of As and Sb in the initial experiments used the lower concentration of Sb (0.012 mM), the increase in inhibition may be due to the increased Sb concentration (0.021 mM) rather than pre-exposure.

IMPLICATIONS

My results show competitive uptake of Sb(III) and As(III) does not affect toxicity in *M. thermautotrophicus* at concentrations relevant to populations at ETGF. As(III)-amended cultures, with and without the presence of Sb(III), showed complete inhibition of methanogenesis and growth at concentrations as low as ~0.05 mM. These results corroborate previous work on As(III) toxicity in methanogens (Sierra-Alvarez et al., 2004).

Although As(V) was tolerated better by *M. thermautotrophicus*, inhibition in growth and CH_4 production was still observed. The abiotic reduction of As(V) to As(III) over time causes complete inhibition if As(III) concentrations exceed ~0.05mM.

Culturing *M. thermautotrophicus* with both Sb(III) and As(V) did mitigate As toxicity by preventing the reduction of As(V) to As(III). More As_T is removed from solution in the presence of Sb(III) as well, suggesting that As precipitation, probably as arsenic sulfide, is stimulated by Sb(III). The presence of Sb(III) also increased the As_T removed from the aqueous phase relative to the sterile control, indicating some biological transformation of As in the presence of Sb may have occurred. Future analysis of culture headspace gas to look for production of methylated Sb and As compounds may be performed to test this hypothesis.

Results suggest that Sb(III) has no inhibitory effects on growth of *M. thermautotrophicus* or methane production, and may improve growth and methane production, particularly in exponential growth phase. However, further testing on the

fate of Sb in these cultures, including the timeline of Sb removal from the aqueous phase, Sb speciation, the presence of methylated Sb compounds, and formation of Sb precipitates is needed. Biomethylation studies have shown *M. thermautotrophicus* can tolerate Sb concentrations of 0.1-0.5 mM, much higher than those used here, although enhanced growth and methane production rates were not previously reported (Michalke et al., 2000).

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Chapter 4: Biodiversity and community ecology at different sites in the El Tatio Geyser Field

ABSTRACT

Diverse microbial mat communities thrive at many of the hydrothermal features at El Tatio Geyser Field (ETGF) despite extreme conditions and the presence of inhibitory toxins such as arsenic (As) and antimony (Sb). High diversity may indicate a widespread resistance to toxic metalloids, high temperatures, high salinities, low carbon concentrations and other challenges present at ETGF. In this case, correlations between specific environmental parameters and microbial community structure and composition may be expected. Alternatively, the formation of microniches may mitigate the extreme conditions and promote diversity. In this case, microbial diversity would be dependent on syntrophic relationships, in which less-resilient microorganisms are protected.

In this study, the beta diversity between four hydrothermal features at ETGF is explored within the context of inhibitory physicochemical parameters to assess which parameters control alpha diversity, shared species, and general microbial ecology. The parameters explored include As and Sb concentration, As oxidation state, temperature, salinity, dissolved methane (CH_4), hydrogen (H_2), sulfate (SO_4^{2-}), and inorganic carbon (DIC).

Results indicate that archaeal and bacterial communities are controlled by different parameters. Bacterial communities show a strong correlation to temperature and

As oxidation state, whereas archaeal communities show weak correlations to H_2 and SO_4^{2-} concentrations. The stronger correlations observed between environmental parameters and bacterial populations may indicate that resistance to specific environmental stresses may control bacteria at ETGF. Archaea do not show strong correlations to environmental stresses, and community composition therefore may depend more on syntrophic relationships with more resistant organisms.

INTRODUCTION

Microbial ecology is a rapidly growing field, fueled by the expanding understanding of the role microorganisms play in global biogeochemical cycling. Rapid accumulation of phylogenetic data from environmental sequences allow for characterization of phylotype richness in a variety of ecological settings. Examining patterns in beta diversity along temporal and spatial gradients suggests environmental controls on community composition. Identifying patterns in community composition and diversity and linking those patterns to causal environmental parameters can be used to catalogue biodiversity within specific ecological settings, and to predict community structure in analogous sites that cannot be easily sampled. Controls on biodiversity include dispersion and other spatial processes, environmental constraints, and niche competition. Patterns in diversity can be used to estimate how rates of evolution of certain lineages vary with environmental settings (Graham & Fine, 2008; Pausas & Verdú, 2010), and may be informative in determining potential metabolic roles of phylogenetic groups within an ecological framework (Auguet et al., 2010).

Beta diversity in microbial community ecology was broadly defined by Whittaker (1960) as "The extent of change in community composition, or degree of community differentiation, in relation to a complex-gradient of environment, or a pattern of environments." Quantitatively, beta diversity is calculated as a function of alpha and gamma diversity; where alpha diversity is the species richness within a given community,

and gamma diversity is the species diversity of a number of community samples from a range of environments.

Over time the concept of beta diversity has taken on numerous meanings, prompting stricter definitions and clearly defined quantitative measures of "beta components" (Tuomisto, 2010). In this chapter I will discuss, the two components of beta diversity defined by Andersen et al. (2011). The first aspect of beta diversity deals with variation in the community structure between categorical factors or sampling units. The second, known as species turnover, measures changes in community structure along an environmental gradient.

In some cases, archaeal and bacterial populations correlate to different environmental parameters (Mueller et al., 2010), suggesting that environmental controls on archaeal and bacterial diversity may differ. Many studies have found that the relative abundance of Bacteria and Archaea may change over geochemical gradients due to competitive processes (Beman & Francis 2006; Nicol et al., 2008); however, these studies focus on populations that directly compete for a metabolic substrate (eg., denitrifiers or ammonia-oxidizers). Additionally, bacterial and archaeal diversity can change along geographic gradients (Braker et al., 2001) although the cause of these changes may be linked to geographic locations or differences in environmental parameters at each site.

In this study, the diversity of Archaea and Bacteria from the El Tatio Geyser Field (ETGF) was analyzed to determine potential environmental constraints on community composition. Four hydrothermal features at ETGF with different geochemical signatures

were used to ascertain correlations between community composition and environmental parameters. Two of these sites are within the same stream system, although several kilometers apart, and two of the sites are situated very close to each other geographically, but are otherwise (surficially) unconnected. Dispersion was not taken to account in this study, because only microbial mats and not placktonic microorganisms were observed, and any changes in community composition or diversity were attributed to differences in environmental parameters.

High alpha diversity was observed previously within Archaea at ETGF (see Chapter 2 of this dissertation) suggesting either that phylogenetically diverse microorganisms are capable of tolerating the extreme conditions there, or that syntrophic relationships between populations within microbial mats allow phylogenetically diverse microorganisms to thrive at ETGF, despite environmental constraints. The formation of microniches, which are created through the coupling of microbial populations with complementary metabolisms or detoxification mechanisms, alter geochemistry within microzones of microbial mat (Paerl et al., 2000), and may add to the alpha diversity.

Microniches also impact beta diversity at ETGF. If beta diversity is comparatively low, this could suggest that the presence of microniches controls biodiversity at ETGF, because community members are shared between sites despite differences in environmental constraints. Conversely, if beta diversity is high, environmental parameters are likely to play a larger role in controlling biodiversity and community composition.

The environmental parameters examined include temperature, salinity, As concentration and oxidation state, Sb concentration, dissolved methane (CH_4), and the availability of substrates such as sulfate (SO_4^{2-}), DIC, and dissolved hydrogen (H_2).

Despite their presence in a wide variety of environmental settings, Archaea are often thought of as extremophiles, and their capacity to cope with extremes such as high temperature is well documented (Chaban et al., 2006; Pikuta et al., 2007). Thermophiles, or organisms with an optimal growth temperature of >50˚C, are ubiquitous within the archaeal phylum Crenarchaeota, and are well-represented within other archaeal phyla as well. Although thermophilic Bacteria are common, I hypothesize that bacterial community composition will be temperature-dependent, while archaeal community composition will not.

Arsenic and antimony toxicity and resistance was shown in both Archaea and Bacteria (Filella et al., 2007; Oremland, 2003), so the beta diversity of both domains may correlate to toxic element concentration. The pentavalent form of As is less toxic than its trivalent counterpart (Cervantes et al., 1994), indicating that the oxidation state may also affect community composition. If metalloid toxicity is a factor controlling microbial community composition, high beta diversity should correlate to large differences in metalloid concentration or oxidation state. If no correlation is observed, horizontal transfer of As-resistance genes may be occurring (Cai et al., 2009), or, detoxification by select community members may prevent metalloids from controlling community composition. Because As and Sb are present in high concentrations at ETGF

(Landrum et al., 2009), I hypothesize that both archaeal and bacterial community composition will correlate to metalloid concentration.

Although I hypothesize that temperature and toxic-element concentration will be the environmental parameters that correlate closely with community composition, the effects of total dissolved solids (TDS), pH, DIC, H_2, CH_4, and SO_4 were tested. With the exception of TDS, these parameters are substrates or byproducts of microbial metabolism, and may illustrate changes in metabolic guilds of microorganisms represented at each site (Boyd et al., 2010; White & Findlay, 1988). In an effort to measure the beta diversity of a specific metabolic guild, sequences identifying with methanogenic Archaea were analyzed separately against all environmental parameters to look for trends.

METHODS

Study site

Located in northern Chile, ETGF imposes unique environmental hardships on microbial communities. Hydrothermal saline waters discharge from geyser and spring features at high temperatures (65-86˚C, the local boiling point) and with high concentrations of toxic elements such as arsenic (As) and antimony (Sb). In addition to tolerating these conditions, microorganisms at ETGF must also endure high UV-A and UV-B radiation (~3x average UV at sea level; Phoenix et al., 2006) and other challenges associated with high altitude, periods of desiccation due to the extremely arid climate, and low concentration of dissolved inorganic carbon (DIC).

Sample location

Four sites from three hydrothermal basins within the ETGF were sampled. Sites were selected based on the presence of microbial mats, and on significant differences in temperature and/or water chemistry that may structure microbial community composition. Sites were described in detail and placed within the context of the hydrothermal field in earlier chapters. Briefly, two of the sites are sourced from hydrothermal springs, one from a hydrothermal pool, and one from a geyser. Physicochemical characteristics of each site are shown in Table 4.1.

The "GG" geyser is characterized by high temperature, salinity, and toxic metalloid concentration, and low H_2 and DIC. The "MBS" spring is slightly more dilute but similar in composition, but with much higher H_2 and DIC. These two features are separated by ~100 m distance from each other (Figure 4.1).

The "UBP" pool is relatively low in temperature (~30˚C), but comparable to the first two sites in salinity, with high As, Sb, and DIC. Sulfate concentrations are much lower at this site than at the other tested sites, and dissolved CH_4 is high. This pool is ~1.3 km north of the GG and MBS sites.

Finally, the "LTS" site is distinct from all other sites tested, in that temperature, salinity, and toxic element concentrations are much lower. This site is situated ~2 km upstream of the MBS site, and water is derived both from both meteoric and hydrothermal sources. Despite the close proximity of the GG and MBS sites, and the connection between the MBS and LTS sites, changes in community composition due to

dispersion was not explored, as planktonic microbial community was not part of the study.

Table 4.1: Physicochemical parameters of 4 hydrothermal sites tested.

Site	source	other inputs	Temp (˚C)	TDS (ppm)	As (ppm)	%As(V)	Sb (ppm)	DIC (ppm)	SO_4 (ppm)	CH_4 (mg/L)	H_2 (ug/L)
GG	geyser	none	65	10000	35	30	2	7	250	0.2	0.08
UBP	pool	none	33	8000	22	100	1.4	30	65	4.5	0.34
LTS	spring	meteoric stream	62	175	0.1	100	0.03	20	200	2.9	0.18
MBS	spring	mixed stream*	33	6000	30	50	1.1	40	175	0.3	0.36

*mixed indicates both hydrothermally sourced and meteoric sourced water

Sample Collection

One microbial mat samples were aseptically collected and stored at 4˚C for no longer than one week before DNA extraction. Total environmental DNA was extracted from 1 mL aliquots of mat-water slurries using a soil DNA extraction kit (mo-Bio Laboratories Inc., Carlsbad CA, USA). Final DNA concentrations were measured spectroscopically (Nanodrop, Thermo Fischer Scientific Inc., Waltham MA, USA), and ranged between 13-50 ng·μl-1.

Amplification and sequencing was performed by Research and Testing Laboratory (Lubbock, TX) on a Roche 454 Genome Sequencer system using standard protocols. A portion of the 16S rRNA gene sequence was amplified using archaeal primers Arch349F (‘5-GYGCASCAGKCGMGAAW) and Arch806R (‘5-GGACTACVSGGGTATCTAAT), and bacterial primers U28F (5’-

AAGAGTTTGATCCTGGCTCAGA) and U519R (‘5- GWATTACCGCGGCKGCTG). A total of 30,740 bacterial sequences of six samples (three from different points of the MBS site) and 15,117 archaea sequences covering the four sites were produced. In direct comparisons of bacterial and archaeal diversity data, only the four sites described here were used.

Processing of pyrosequencing data

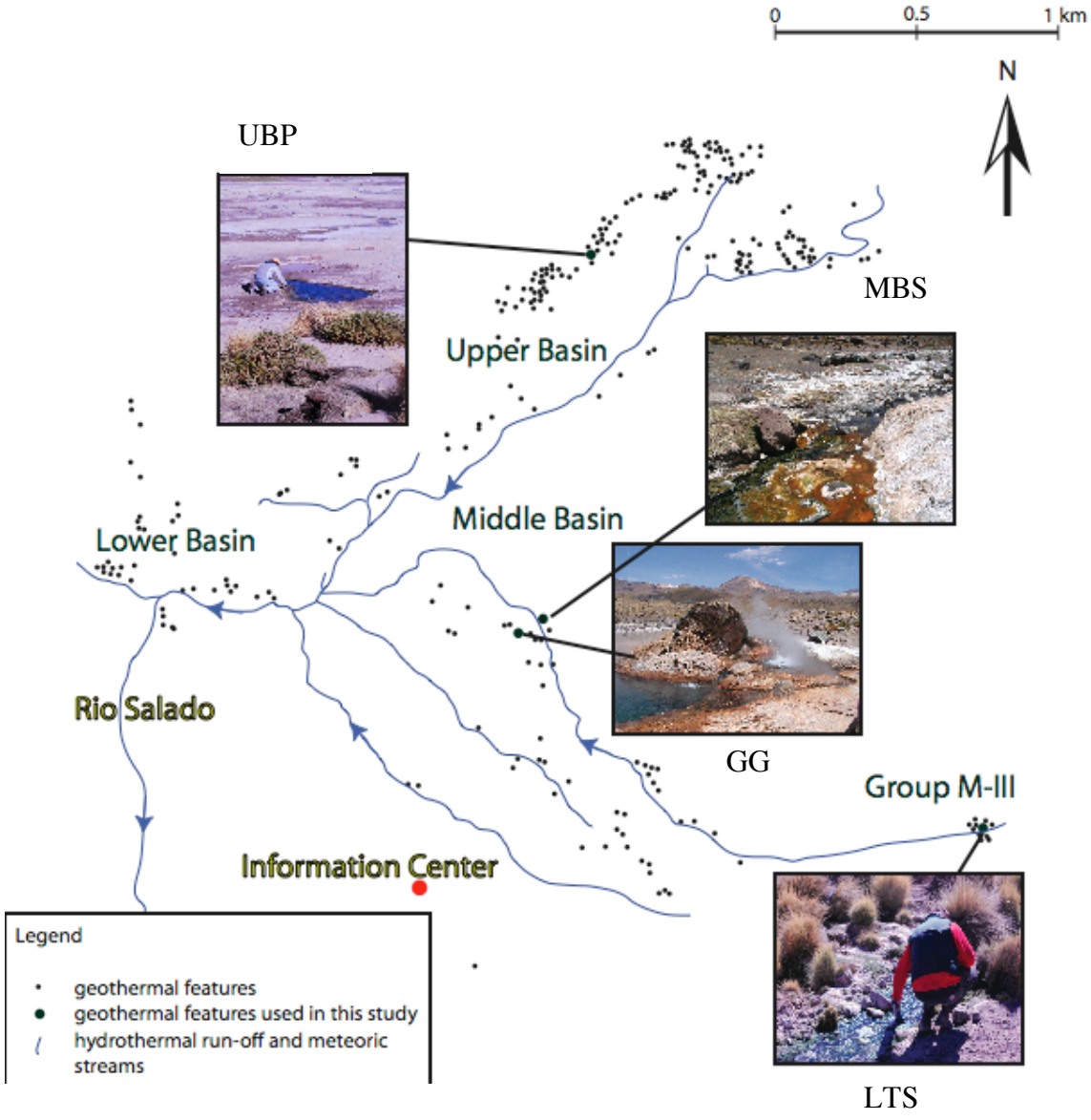
Bacterial and archaeal sequences were analyzed separately. All sequences were screened for quality, and sequences containing ambiguous base pairs or homopolymers longer than eight base pairs were removed from analysis. Sequences were aligned using the greengenes reference alignment and the align.seqs function in MOTHUR (Schloss et al., 2009), which uses the Needleman-Wunsch pairwise alignment method. Seque nces were checked for putative chimeras using the greengenes gold alignment and the chimera.slayer function, and chimeras were removed from analysis.

Phylotypes: OTU identification, diversity and estimated richness

Phylip-formatted distance matrices for Bacteria and Archaea were constructed in MOTHUR using the dist.seqs function. Operational taxonomic units (OTUs) were determined using similarities of 90 and 97%, corresponding to putative family and species level divisions (Hur & Chun, 2004), using the furthest-neighbor algorithm.

Two non-parametric richness indices, Chao 1 and the abundance-based coverage estimator (ACE), were used to calculate a lower limit of alpha diversity at each site.

Figure 4.1: Sample locations.



These indices estimate the total number of OTUs present at each site. Simpson's index, a diversity measure that focuses on the evenness and dominance of the sample, gives the probability that two clones drawn at random will be from the same OTU. Because both estimators vary with sample size, samples were normalized to the size of the smallest sample set prior to analysis (Lemos et al., 2011). All calculations for diversity measures were done in MOTHUR.

Analysis of community structure and diversity

The overlap of observed OTUs between the four sites were compared using 90% and 97% similarity and represented graphically using a Venn diagram. Taxonomic classification of OTUs and other beta diversity measures use the 90% OTU (family) cut-off.

Beta diversity was explored here using multivariate statistical analysis showing the significance of correlation between environmental parameters and community dissimilarity. The relationship between alpha diversity (as defined by Simpson's index) and each environmental variable was examined using linear regression. In addition to Bacteria and Archaea, archaeal sequences that classified within a methanogenic order (≥80% similarity) were analyzed to see if trends in beta diversity could be more easily observed.

Beta diversity of variation between the four sites is represented graphically in non-metric multidimensional scaling (NMDS) plots. Distance matrices using both the Jaccard distance between each site, which is used to assess dissimilarity in community

membership (Whittaker, 1960), and the Yue & Clayton theta coefficient, used to assess differences in community structure (Yue & Clayton, 2005), were used to generate plots. Normalized data sets were used for each analysis.

Dissimilarity was statistically related to relevant environmental variables (see Table 4.1) using analysis of similarity (ANOSIM), which ranks the similarity of groups to determine if they are more similar than random groups (Clarke, 1993), and analysis of molecular variance (AMOVA), which tests the genetic diversity between populations to determine if it is significantly different from the diversity resulting from pooling the populations (Excoffier et al., 1992). In order to perform these analyses using only one sample per site, the parameters tested were categorically defined as high, medium, and low for comparative purposes (Table 4.2). These analyses were performed for the Bacteria, Archaea, and methanogen datasets using Jaccard distances.

Table 4.2: Categorical definitions of relevant environmental parameters used in multivariate analyses.

Site	source	other inputs	Temp	TDS	As	%As(V)	Sb	DIC**	SO_4	CH_4	H_2
GG	geyser	none	high	high	high	low	high	7	high	low	low
UBP	pool	none	low	high	low	high	med.	30	low	high	low
LTS	spring	meteoric stream	high	low	med	high	low	20	high	high	low
MBS	spring	mixed stream*	low	high	high	low	med.	40	high	low	high

*mixed indicates both hydrothermally sourced and meteoric sourced water
**DIC was not used in categorical analyses

Linear regressions relating alpha diversity to environmental parameters were used to determine correlations between the normalized alpha diversity of each hydrothermal site and physicochemical variables.

RESULTS

Pyrosequencing and OTU definition

After quality checks, definition of unique sequences, trimming, alignment and chimera checking, 13,410 bacterial sequences and 12,584 archaeal sequences were used for OTU definition. A total of 920 archaeal sequences are closely related to known methanogenic archaea.

Of the bacterial sequences, 4233 sequences were classified within the phylum Proteobacteria, 1552 sequences within Deinococcus-Thermus, 872 within Firmicutes, 829 sequences within Flavobacteria, and 643 within Cyanobacteria. Of the archaeal sequences, 7433 were classified as Crenarchaeota, 2372 Euryarchaeota, 1034 Korarchaeota, and 533 Nanoarchaeota.

At the 90% similarity cut-off, 211 abundant OTUs (>10 sequences) were found in Bacteria, and 101 abundant OTUs were found in Archaea. The taxonomic affiliation of the most abundant OTUs can be found in Appendix C. The most abundant OTU in the Bacteria was Meiothermus, a genus of the order Deinococcus-Thermus; and the most abundant archaeal OTU identified with the crenarchaeal family Thermoproteaceae.

Coverage and Alpha diversity

Alpha diversity varied among sites, with the highest estimated richness at the low temperature sites (UBP and LTS). Diversity values, including Simpson's diversity index, estimated species richness, and observed OTUs were normalized to the smallest sample size (Table 4.3): 1530 sequences at the UBP site for Archaea, and 1006 sequences at the GG site for Bacteria. Both the Chao index, which is indicative of minimum species richness at each site, and ACE values suggest that richness is highest for the UBP site, and second highest at the LTS site, at the 90% OTU similarity cut-off. Using a 97% OTU similarity cut-off suggests that the total richness of the LTS site is greater for Archaea, while for Bacteria the UBP site still has the highest richness. The difference in archaeal species richness based on the chosen OTU cut-off suggests that significant evolutionary differentiation has taken place within archaeal "families" in the LTS site.

Table 4.3: Diversity of each site. Sobs: observed OTUs; ACE: abundance-based coverage estimator; CHAO: Chao1 shared species estimator; D: Simpson's Diversity Index. Calculations are normalized to prevent differences in microbial diversity based on sample size.

	Bacteria					Archaea				
0.1	Sobs	ACE	CHAO	D	coverage	Sobs	ACE	CHAO	D	coverage
GG	136	320	283	0.07	0.93	85	146	132	0.101	0.98
UBP	544	3967	1622	0.01	0.64	114	233	216	0.058	0.97
LTS	436	1966	1504	0.01	0.67	109	216	152	0.141	0.97
MBS	242	827	536	0.06	0.84	57	111	93	0.154	0.98
0.03										
GG	271	718	531	0.02	0.84	323	475	499	0.01	0.91
UBP	758	7721	3361	0.003	0.47	274	902	636	0.03	0.89
LTS	501	2175	1786	0.002	0.55	497	1388	976	0.01	0.81
MBS	348	1340	1025	0.03	0.71	240	488	366	0.02	0.92

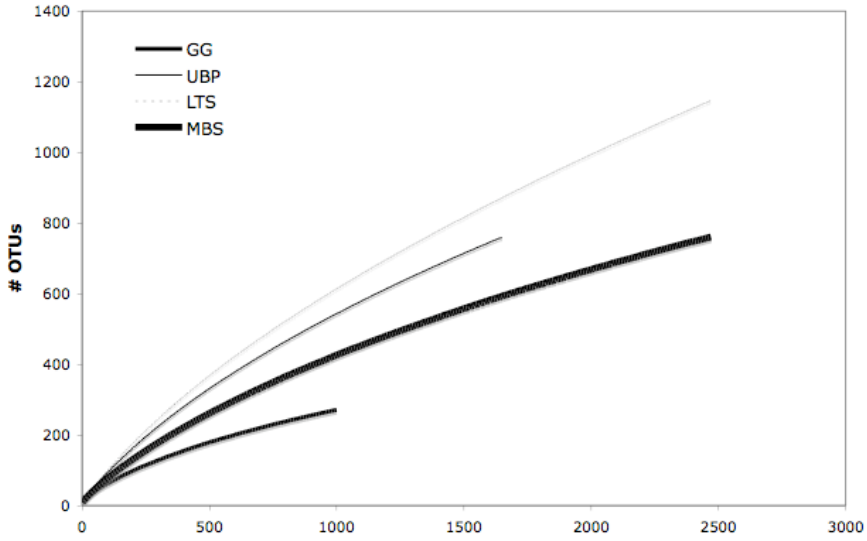
In the previous study, the alpha diversity of Archaea was high within the MBS site, and low in the UBP site (Chapter 2). This may be indicative of differences in the sequencing process (Sanger vs. pyrosequencing) or in primer selection. Alternatively, the fact that data in that study was not normalized to the sampling effort may have skewed original diversity data. In this study, the MBS site had the lowest species richness and diversity.

The UBP and LTS sites are low-temperature compared to the GG and MBS sites, suggesting species richness may be related to temperature at ETGF. Simpson's diversity index was also highest for the UBP and LTS sites for Bacteria. In archaeal communities, Simpson's diversity index was highest for the UBP site and the GG site. Despite the GG site having relatively low species richness, the diversity was higher than that of the LTS site or the MBS site. This suggests that the GG site has a high number of rare OTUs.

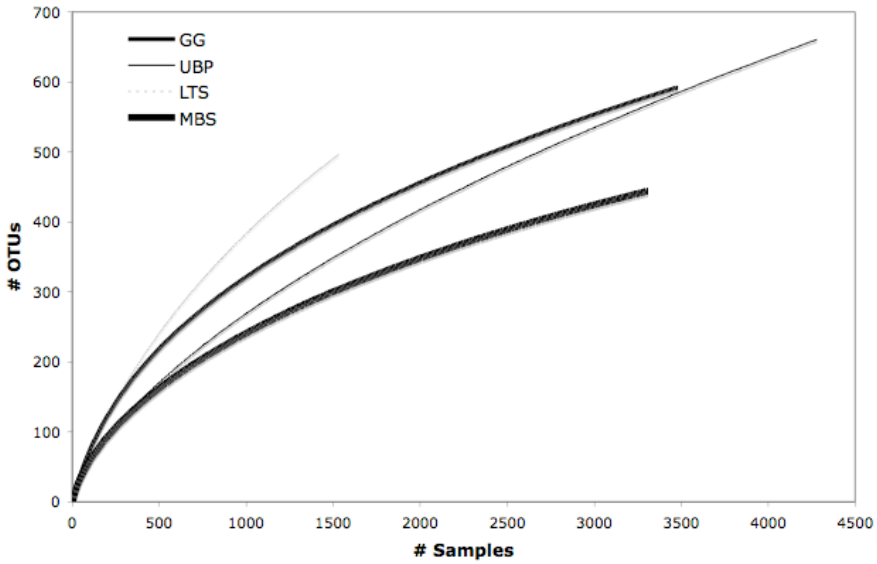
Rarefaction curves for Bacteria and Archaea at the 90% cut-off similarity (Figure 4.2) show the total number of OTUs, rather than normalized values (Table 4.3). A disparity in the number of sequences sampled for each site illustrates the need for normalized diversity calculations. Rarefaction curves agree with diversity indices, and indicate that the two low-temperature sites, the UBP and LTS sites, are more species-rich than the high-temperature sites (GG and MBS).

Figure 4.2: Rarefaction curves for (a) Bacteria and (b) Archaea.

(a) Bacteria



(b) Archaea



Shared Species and Beta Diversity

In general, few taxa were shared at the 97% similarity level for both Bacteria and Archaea (Figure 4.3). At the 90% similarity level, most bacterial OTUs are shared between the GG and the MBS site and the UBP and LTS sites. Archaeal OTUs do not share this pattern, and most taxa are shared between the LTS and GG site. This may be indicative of different physicochemical properties dictating archaeal and bacterial community composition.

Typically, abundant OTUs (defined here as OTUs containing ≥10 sequences) are most often shared between sites. However, at the 90% similarity cut-off, only 34 of the 92 shared bacterial OTUs and 62 of the 101 shared archaeal OTUs are abundant.

For Bacteria, the shared taxa are representative of all shared OTUs (i.e., abundant and rare OTUs overlap phylogenetically), so that all shared phylogenetic groups are shown (Table 4.4). The order Thermales is the most common shared taxonomic group, other than unclassified Bacteria.

Archaeal OTUs are more likely to be shared between multiple sites than bacterial OTUs, and multiple taxonomic groups are shared between sites (Table 4.4, Figure 4.3). OTUs identifying with the class Thermoprotei (including the order Thermoproteales within this class) are shared most often (excluding unclassified Archaea.) Few euryarchaeal OTUs are shared between sites as compared to Crenarchaeota. Those that are shared are rare OTUs identifyied with the orders Methanomicrobiales, Methanobacteriales, Methanopyrales, and Methanosarcinales. Numerous archaeal OTUs

were identified as Nanoarchaeum and Korarchaeum, but were rare in abundance and seldom shared between sites.

The number of Korarchaeota at these ETGF sites is high in comparison to many hydrothermal systems (Auguet et al., 2010). Korarchaeota are a deeply branching clade that diverged before the separation of Crenarchaeota and Euryarchaeota (Barns et al., 1996; Elkins et al., 2008). Originally isolated from terrestrial hot springs at Yellowstone National Park (Barns et al., 1994), they have since been discovered at a number of hydrothermal sites, including hot springs in Iceland (Hjorleifsdottir et al., 2001) and Kamchatka, Russia (Reigstad et al., 2010), deep sea vents (Nercessian et al., 2003) and sulfide chimneys (Schrenk et al., 2003). Previous authors showed that the clades formed by Korarchaeota are highly influenced by geographic isolation (Auchtung et al., 2006), suggesting that the korarchaeal population at ETGF may be a new phylogenetic order. A low % identity within BLAST (Appendix C) supports that hypothesis.

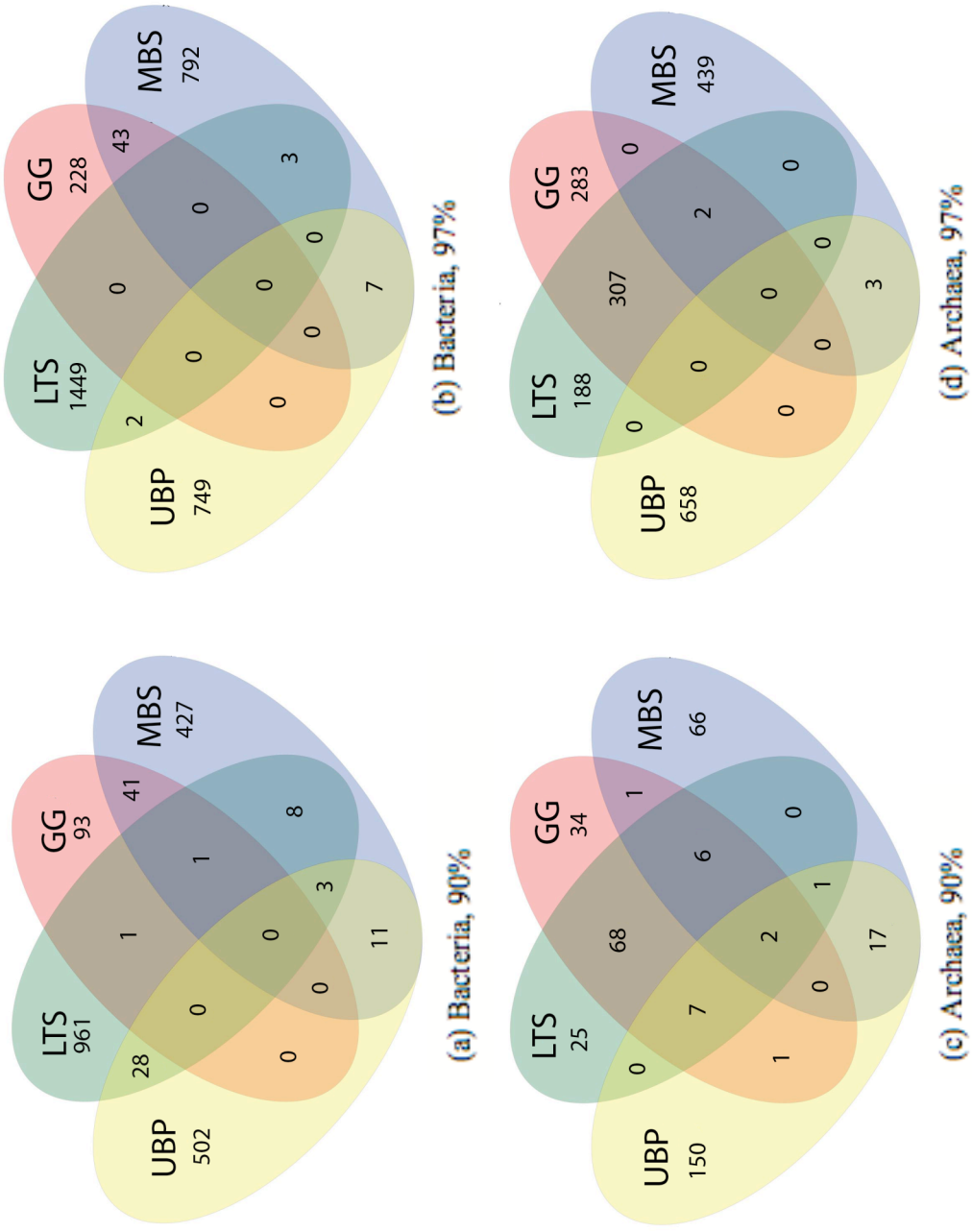
The grouping of sites on NMDS plots is similar using both the Jaccard and Yue and Clayton index (Figure 4.4). Site grouping is different for Bacteria and Archaea, suggesting different parameters are responsible for structuring archaeal and bacterial communities.

In bacterial analysis, the UBP and LTS sites group together, and the GG and MBS sites group together (Figure 4.4a). I interpret those differences in grouping as temperature-driven. Analysis using the Yue and Clayton coefficient suggests that the GG site is distinct from the other three sites tested.

Table 4.4: Taxonomic Identification and number of abundant OTUs shared between sites at 90% OTU ID.

	Taxonomic affliiation	GG & LTS	GG & MBS	LTS & MBS	MBS & UBP	LTS & UBP	GG, LTS, MBS	GG, LTS, UBP	GG, UBP, LTS, MBS
Bacteria	Acidobacteriales			1					
	Betaproteobacteria		2						
	Burkholderiales				1				
	Caldilineales		1						
	Chloroflexales		1						
	Desulfuromonadales				1				
	Flavobacteriales			1					
	Hydrogenophilales		1						
	Oscillatoriales					1			
	Rhodobacterales			1	2	1			
	Rhodocyclales					1			
	Sphingomonadales					1			
	Thermales	1	3	0			1		
	Unclassified Bacteria		9	1		2			
	Verrucomicrobiales					1			
Archaea	Thermoproteales	5			5		1		2
	Euryarchaeota	2						1	
	Archaea	14			5			3	
	Methanococcales	2					1		
	Desulfurococcales	1			1		1		
	Thermoprotei	7	1		3		2	1	
	Methanomicrobiales	1							
	Nanoarchaeum				1				
Eukaryota	Bacillariophyta					1			

Figure 4.3: Venn diagrams showing shared OTUs for Bacteria at (a) 90% OTU similarity and (b) 97% OTU similarity, and Archaea at (c) 90% and (d) 97% similarity.



Because the GG site is high in temperature, highest in dissolved elements, and has limiting DIC and H_2, a distinct community may also be expected there.

The archaeal NMDS plots indicate that the GG and LTS community composition and structure group together, and suggest that the MBS and UBP sites are more similar to each other than to either the LTS or GG sites (Figure 4.4b). Dissimilarity of methanogenic populations (4.4c) between sample sites deviates little from analysis of the domain Archaea as a whole, showing the GG and LTS sites group together, and the MBS and UBP sites group.

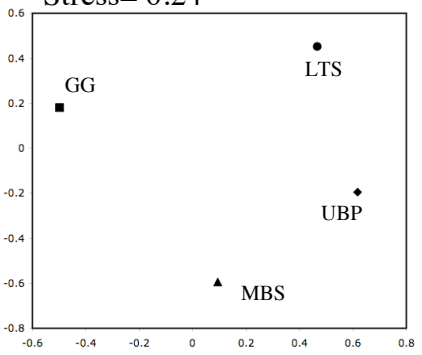
These groupings differ from what was observed with bacterial populations, and suggest that temperature does not drive archaeal community structure at ETGF features. The GG site and the LTS site are different in terms of environmental parameters, indicating either that archaeal communities at ETGF do not correlate to water temperature or chemistry, or that another geochemical parameter is dictating community composition. The only shared geochemical characteristics are the relatively high SO_4 concentrations and relatively low H_2 concentrations at these two sites.

Unfortunately, due to the lack of replication in this study, I was unable to perform statistical analyses that could confirm these speculations.

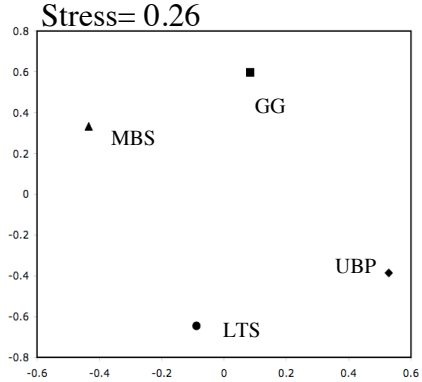
Results from ANOSIM analyses did not demonstrate any correlations between environmental parameters and observed beta diversity. Results from AMOVA analysis suggested three environmental variables were significant (Table 4.5). ANOSIM converts similarities into ranks, which decreases the amount of information included in the analysis, because differences in the amount of variability are not detected.

Figure 4.4: NMDS plots for Bacteria, Archaea, and methanogenic Archaea. Jaccard distances are used in (a) plots, and Yue & Clayton distances are used in (b) plots. Samples are represented: (■) GG, (●) LTS, (▲) MBS, and (◆) UBP.

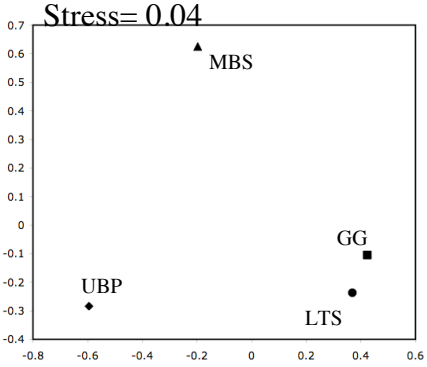
(1a) Bacteria: Yue & Clayton
Stress= 0.24



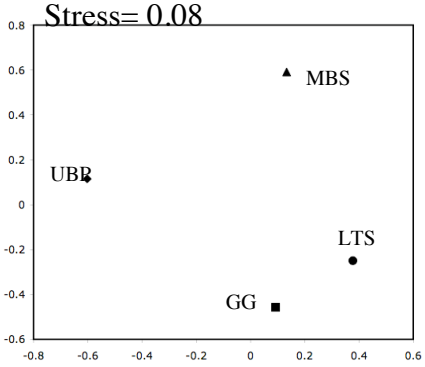
(1b) Bacteria: Jaccard
Stress= 0.26



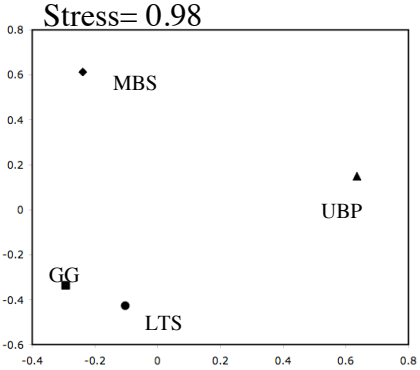
(2a) Archaea: Yue & Clayton
Stress= 0.04



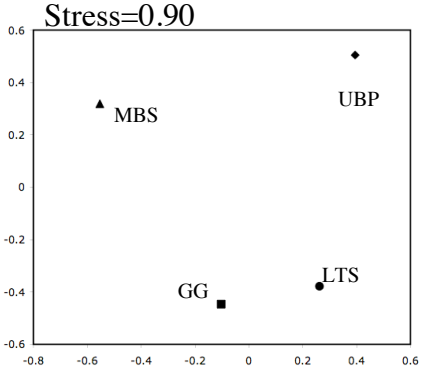
(2b) Archaea: Jaccard
Stress= 0.08



(3a) Methanogens: Yue & Clayton
Stress= 0.98



(3b) Methanogens: Jaccard
Stress=0.90



In AMOVA analysis, molecular data is used to determine similarities. This method incorporates more information into the analysis and thus it was able to distinguish significant relationships between environmental variables and community composition. AMOVA indicates temperature-structured bacterial communities across sites ($p<0.0001$), and both dissolved hydrogen concentration and sulfate concentration affected archaeal species turnover ($p<0.0001$ for each). Data for analysis of methanogen populations only are not shown, because results match those observed for archaeal analysis.

The relationship between environmental parameters and diversity at each site was also explored using classic linear regression analysis. Figure 4.5 shows relationships between environmental parameters and Simpson's alpha diversity. All the parameters shown in Table 4.5 were analyzed; however, only the environmental variables shown in Figure 4.5 had significant r^2 values.

Whereas only temperature was significantly related to variations in bacterial community composition between sites, bacterial alpha diversity correlates strongly with both water temperature ($r^2=0.992$, $p=0.004$) and the percentage of dissolved arsenic in the As(V) valence state ($r^2=0.996$, $p=0.002$). A correlation between alpha diversity and dissolved methane concentration was also observed ($r^2=0.891$, $p=0.056$).

No significant correlations between archaeal alpha diversity and environmental parameters were observed; however, r^2 values suggest dissolved hydrogen ($r^2=0.866$, $p=0.134$) and DIC ($r^2=0.818$, $p=0.182$) may be important environmental parameters.

Table 4.5: Significance of environmental variables in explaining beta diversity.

Variable	Range	Archaea p-value*		Bacteria p-value*	
		AMOVA	ANOSIM	AMOVA	ANOSIM
Temperature	30-65˚C	0.673	1	<.0001	0.066
Salinity (Na+Cl)	170-10000 ppm	0.734	1	0.081	0.167
Arsenic	0.1-35 ppm	0.837	1	0.155	0.227
As(V) %	20-100%	0.495	0.668	0.114	0.066
Sb	0.03-0.2 ppm	0.164	0.338	0.369	0.315
H2	0.1-1 umol/L	<.0001	0.161	0.279	0.74
CH4	13-280 umol/L	0.665	1	0.461	0.198
SO4	60-250 ppm	<.0001	0.168	0.26	0.399

*p-values calculated from normalized Jaccard distance matrices.

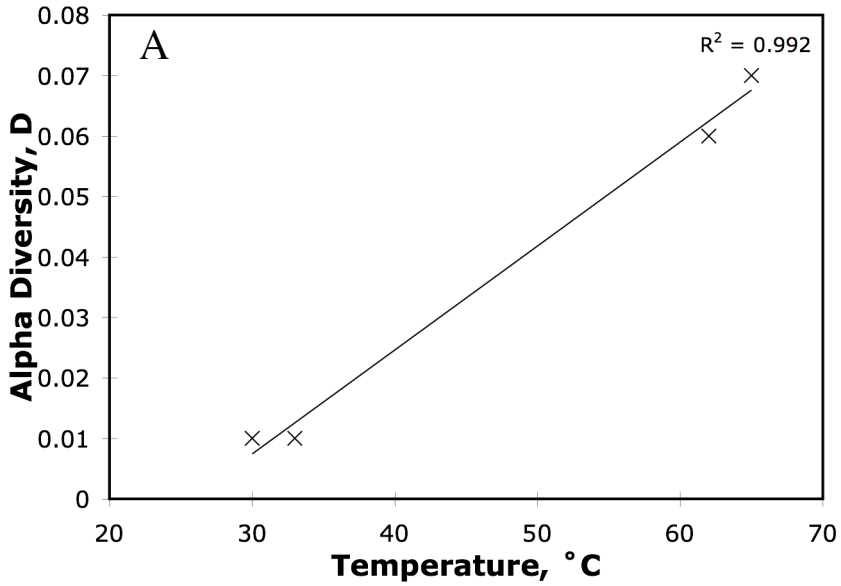
DISCUSSION

Temperature

The similar community membership and lower diversity at high-temperature sites suggests that limited bacterial populations are capable of tolerating high temperatures in conjunction with other extreme ETGF conditions. The relationship between bacterial community and temperature is clearly shown with Jaccard dissimilarity, indicating that community membership, rather than structure, differs between high- and low-temperature sites. Greater diversity at lower temperatures The AMOVA analysis of Bacteria supports a significant relationship between community membership and temperature.

No relationship was observed between specific bacterial taxa and temperature (data not shown). An OTU that classified as Thermales, a thermophilic order of Bacteria, was shared between one of the high- and one of the low- temperature sites; and other representatives of Thermales were found at both low-temperature sites. Thermophilic microorganisms are often found in a wide range of temperatures, however.

Figure 4.5: Linear regression results for environmental parameters showing correlation to alpha diversity measurements. Bacterial alpha diversity correlates with (A) temperature, (B) % As(V) present, and (C) CH_4 concentration.



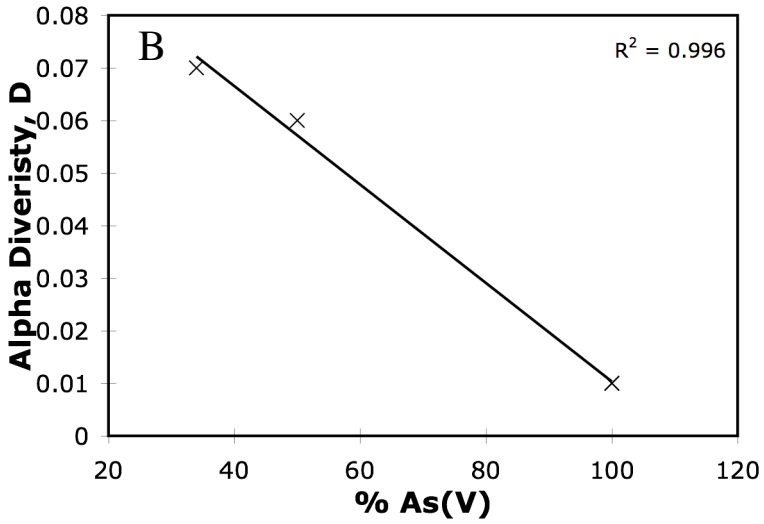
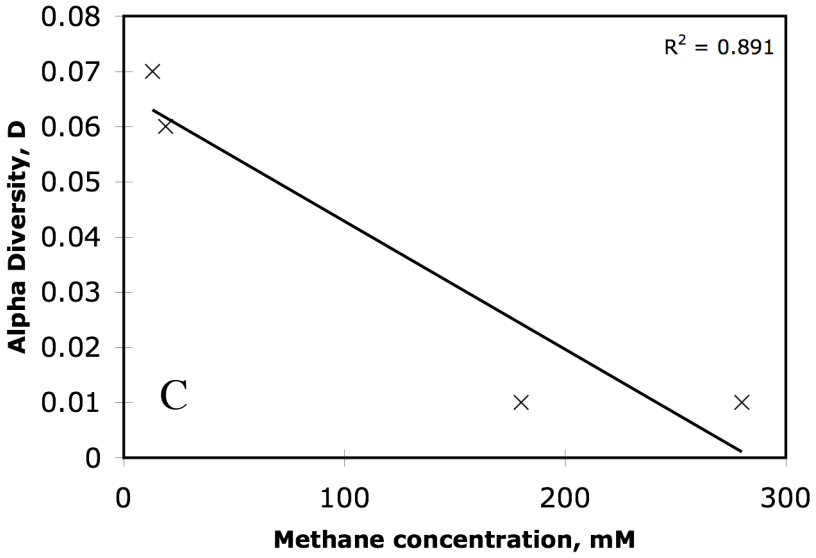


Figure 4.5 con't: Bacterial alpha diversity correlates with (C) CH_4 concentration. Diversity of methanogenic Archaea shows weak correlation with (D) dissolved H_2.



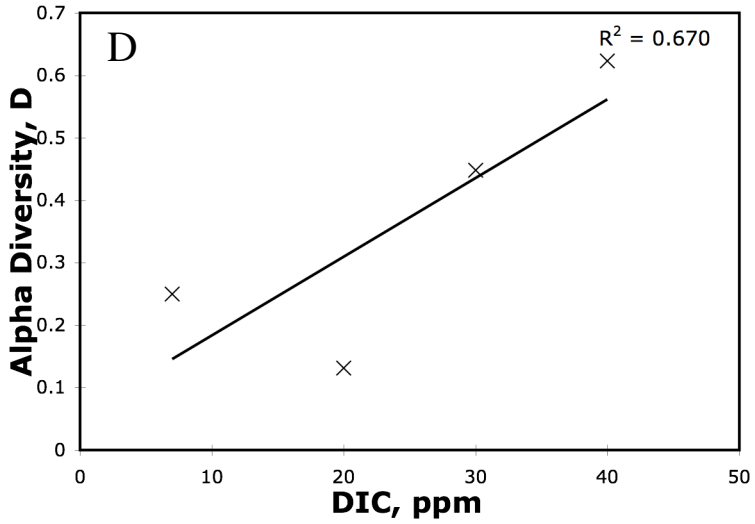
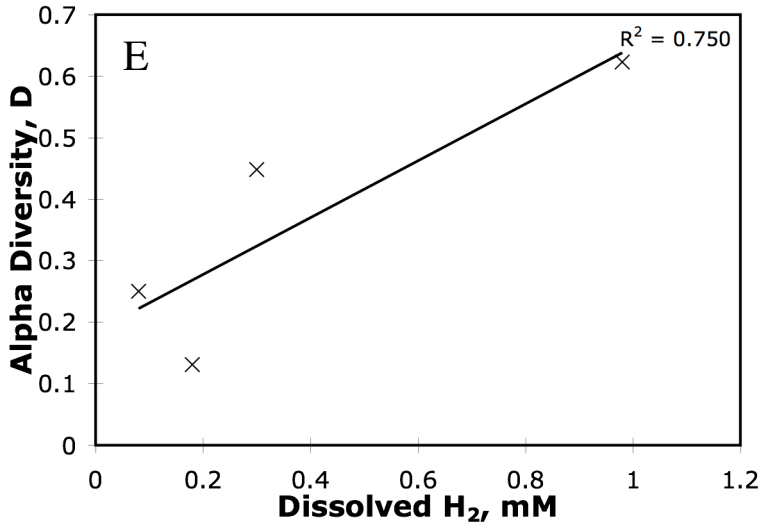


Figure 4.5 con't: Diversity of methanogenic Archaea shows weak correlation with (E) DIC.



There are several explanations for the presence of Thermales at high- and low-temperature features. This may be evidence of changes in hydrothermal activity at ETGF over time creating different physicochemical conditions at each site, followed by evolutionary adaptation of the thermophilic microorganism to be competitive at a lower-temperature site. Alternatively, this may be indicative of the dispersion of thermophilic microorganisms to lower temperature sites.

For Archaea, including the subgoup of methanogenic Archaea, the water temperature was not statistically significant in determining dissimilarity between sites. Methanogenic Archaea are typically found in moderate temperatures, although their range extends from psycrophilic to hyperthermophilic environments. Similarly, the diversity of Archaea and methanogenic Archaea did not correlate to temperature, suggesting that this parameter had little influence on archaeal communities.

The correlation between bacterial community diversity and membership and temperature indicates that temperature strongly influences bacteria, and may indicate that microzones in microbial mats may not alter temperature significantly enough to protect temperature-sensitive bacterial taxa. Archaeal communities appear unaffected by differences in temperature, which suggests that the taxa present are capable of living within a wide temperature range, or at least that thermophilic and mesophilic members of each OTU "family" are present.

Abundant Thermoprotei OTUs were also shared between the sites, despite the fact that Themoprotei are typically found in high-temperature waters, suggesting that the taxa adapted for high temperatures are still present in low-temperature hydrothermal areas.

Hydrothermal metalloid concentration and oxidation state

Neither community composition nor diversity of Bacteria and Archaea showed significant correlation to arsenic or antimony concentrations, despite the inclusion of both extremely high and low concentration sites in the analysis. Indicator species analysis failed to identify OTUs that were strongly affected by total As or Sb concentration (data not shown), and regression analyses indicated that no significant correlation between metalloid concentration and diversity. This suggests resistance to these metalloids may be widespread, due either to convergent evolution or horizontal gene transfer (HGT) (Cai et al., 2009). Convergent evolution is common in arsenic resistance mechanisms (Mukhopadhyay et al., 2002), and Sb-resistance may be similarly transferred between microbial populations.

These results indicate that the microbial community at the LTS site, which contains negligible concentrations of both metalloids, either has the same detoxification capacity as those organisms at high-concentration sites, or that these organisms are easily able to get resistance through HGT. Another possible conclusion could be that syntrophic relationships with detoxifying microorganisms could prevent Bacteria and Archaea lacking resistance mechanisms from being inhibition at high concentrations. In either case, the hypothesis that high concentrations of hydrothermal metals would play an

important role in structuring microbial communities at ETGF was not observed in my data.

The correlation between bacterial communities and the percent of As present as As(V) indicates that the oxidation state of the As does affect community membership and diversity. Only four sample sites are included, and high As(V) percentage is linked to low temperature at these sites, so this may be misleading. Further analysis, including more sample sites, is necessary to confirm this relationship.

Hydrogen and sulfate

Temperature and the oxidation state of As were the only two environmental parameters tested that showed a significant relationship to bacterial communities, and neither of those parameters showed any correlation to archaeal communities.

The surprising likeness in archaeal communities at the LTS and GG sites indicates that the water temperature and total dissolved solids are unimportant in archaeal diversity. Significant correlation between archaeal community similarity and the dissolved H_2 and SO_4 concentrations suggests the competitive metabolic processes are responsible for shaping archaeal communities at ETGF. This is distinct from what was seen in bacterial populations, in which no metabolic substrates was significant in membership or structure.

Methanogenic Archaea compete with sulfate-reducing Bacteria for hydrogen. The correlation of archaeal, but not bacterial, communities to this common metabolic substrate may indicate that a higher percentage of the archaeal community may rely on

hydrogen, or that bacteria are more diverse in their metabolisms. Many Bacteria found at ETGF are closely related to organisms which can metabolize with a variety of heterotrophic pathways, suggesting that hydrogen concentrations are not important to the majority of Bacteria.

This suggests that Archaea with a hydrogen-dependent metabolism may be important in determining archaeal community structure. Analysis of methanogenic Archaea showed the same significant relationship, and suggests that methanogenic populations influence the overall archaeal community composition and structure.

Competition with sulfate-reducing bacteria for H_2 often affects the ability of methanogens to metabolize (Lovley, 1985), as sulfate reduction is a thermodynamically more favorable process than methanogenesis. Thus, the observed correlation between methanogenic (and archaeal) communities and H_2 could be indicative of a H_2 threshold for methanogenesis and sulfate reduction to occur. The fact that archaeal community composition also correlates with SO_4 concentration further supports this hypothesis. The link between SO_4 concentration and archaeal communities may indicate that the sulfate-reducing community at ETGF is composed primarily of Archaea.

Sulfide concentrations in ETGF waters are relatively low, ranging from 0.03-1.18ppm, despite H_2S emissions at concentrations of 100-4200μmol/mol at fumaroles and boiling pools (Tassi et al., 2005). This suggests a sulfur oxidizing community of prokaryotes may be present. Numerous Betaproteobacteria, including known sulfide oxidizers such as *Tepidimonas*, were identified at ETGF sites, and may remove H_2S produced by hydrothermal activity and sulfate reducing populations.

Linear regression showed no correlation between archaeal alpha diversity and environmental parameters. However, linear regression on methanogenic alpha diversity showed a weak correlation with both DIC (r^2=0.67) and H_2 (r^2=0.75). Because hydrogen concentration is important to determining the thermodynamic favorability of methanogenesis, and CO_2 is utilized in hydrogenotrophic methanogenesis, and occurs as a byproduct of acetoclastic methanogenesis, a correlation between diversity of methanogenesis and the availability of substrates is not surprising. While the majority of methanogenic sequences and OTUs classified within the hydrogenotrophic order Methanococcales, some acetoclastic genera including Methanosaeta are present.

Only 18% of the archaeal sequences I analyzed are classified as Euryarchaeota. This is unusual for hydrothermal systems, where Euryarchaeotes from the orders Thermococcales and Thermoplasmatales typically dominate (Auguet et al., 2010). The overlap between methanogenic and archaeal community analysis indicates that community membership and structure may be influenced greatly by metabolic groups that do not make up a large proportion of the community.

Only four abundant shared OTUs were classified as methanogenic Archaea. Three of them were shared between the GG and LTS sites, and one between the GG, LTS, and MBS sites. This further corroborates the hypothesis that methanogenic populations are responsible for the overall similarities in beta diversity observed between the GG and LTS sites.

Other environmental parameters

Because sulfate-reducing bacteria (SRB) and methanogenic Archaea are metabolic groups that have the potential to shape microbial community membership, SO_4 and CH_4 should correlate to microbial community composition and diversity. However, my results do not show those relationships. This supports the hypothesis that the predominant sulfate-reducing community at ETGF is composed of Archaea rather than Bacteria. The lack of correlation found between CH_4 concentration and archaeal communities may indicate that the fate of CH_4 is variable in different hydrothermal features, or that not all of the observed methane is biogenic in nature.

The lack of correlation may suggest that these environmental parameters are not important because microniches in mats cause large variations in SO_4 and CH_4 concentrations, making relationships between overall communities difficult to discern.

CONCLUSION

The environmental parameters influencing bacterial and archaeal communities differ. While this study was limited in scope, results indicate that variation in bacterial and archaeal community structure may correlate to different environmental variables.

No distinction was made in this study as to the affects of environmental parameters and dispersion. The high beta diversity between sites may indicate limited dispersion at ETGF, particularly with regard to mat communities. The presence of thermophilic microorganisms in relatively cool water may be indicative of changes in the hydrothermal field over time leading to adaptations in microorganism. Alternatively,

dispersion could be an important process; but beta diversity remains high because environmental parameters are so important in determining which microorganisms can survive.

Temperature was most important in dicatating bacterial community structure, while it was not significantly correlated to archaeal community structure. Archaeal communities correlated to metabolic substrates such as hydrogen and sulfate. This difference suggests that Bacteria are able to out-compete Archaea metabolically, as long as environmental conditions allow Bacteria to thrive.

These hypotheses should be further explored, particularly by increasing sampling to include hydrothermal features with a wider range in values for the geochemical parameters tested. If results are supported, and the environmental conditions at each hydrothermal feature supports its own unique microbial community, then novel microorganisms could potentially be found in each new hydrothermal feature discovered.

The richness in microbial community membership at each site suggests that diverse microorganisms are capable of tolerating the extenuating conditions at ETGF, and that horizontal transfer resistence genes, or convergent evolution, is responsible for the presence of such diversity. Alternatively, some members of the microbial community may make the environment habitable for less-resistent microorganisms. Further work in this area could help predict how easily microorganisms could colonize contaminated environments.

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Conclusions

Although microbial communities at hydrothermal systems such as Yellowstone National Park have been extensively studied, cataloging novel microorganisms and characterizing the microbial communities at ETGF is a relatively new endeavor. The first microbial ecosystems may have lived in environments similar to that found at ETGF. By determining the diversity present at ETGF, and looking for variations in community diversity with geochemical parameters, I hoped to make inferences about how the first microbial communities were structured.

The alpha diversity for Archaea at ETGF is high, and novel taxa were found within several archaeal lineages. Representatives from all braches of Archaea, including the proposed taxa Nanoarchaeota, Korarchaeota, and Thaumarchaeota, were found there. The high diversity may indicate that resistance to the extreme conditions at ETGF is easily conferred from one taxon to another, or that microniches within microbial mats protect less-resistant taxa.

Diverse methanogenic Archaea were found at ETGF. While these microorganisms are associated with hydrothermal systems, until recently they have not been the focus of microbial studies. Now, the importance of Archaea in geochemical transformations has been recognized (Nicol & Schleper, 2006). The role Archaea played in geochemical cycling on the early Earth, and the role methanogenic Archaea would have played in carbon cycling and adding methane to the atmosphere, makes the study of

methanogens particularly relevant in terrestrial hydrothermal systems that are analogous to early Earth conditions.

The ability of methanogenic Archaea to survive in a system with abundant trivalent arsenic at concentrations that are known to be inhibitory suggests that methanogens at ETGF either are uniquely adapted to cope with the extreme conditions there, or that syntrophic relationships between methanogens and detoxifying (i.e., arsenic oxidizing) microorganisms exist. The lack of correlation between arsenic concentration and oxidation state and methanogenic populations suggests that syntrophic relationships may be more important in methanogen survival. Alternatively, antimony may counteract some arsenic toxicity in the field.

The diversity and community composition of Archaea only showed weak correlations to environmental parameters, suggesting that overall microbial community composition may be more important than geochemical conditions. Bacterial populations showed strong correlations to both temperature and the oxidation state of arsenic, suggesting that the ability of bacteria to withstand these environmental extremes may be important determinants of bacterial community composition.

Appendices

APPENDIX A

Supplementary material for Chapter 2. BLAST identification by sample location and archaeal order. If a % is included in the archaeal classification (*), it indicates the similarity of the nearest relative to the given classification. No % indicates a 100% match.

Phylogenetic affiliation	Accession #	Closest relative	Sequence similarity	MBS	GG	LTS	UBP	C	Example on tree
Unclassified Archaea									
Archaea	DQ836881.1	coastal sediment clone BSa13	99			1			LTS-33
Unclassified Crearchaeota									
Crenarchaeota	AB301861.1	hydrothermal clone pIta-HW-7	89	7					MBS-15
Crenarchaeota	AF255608.1	hot spring clone SRI-298	85-86	3					MBS-8
Crenarchaeota	AY861952.1	Yellowstone clone OPPD016	95-96		2				GG-10
Crenarchaeota* (99%)	AB237757.1	fault clone HDBW-WA24	99-100			7			LTS-12
Crenarchaeota* (98%)	DQ302468.1	Indian soda lake clone LR-352	100			1			LTS-8
Crenarchaeota* (97%)	DQ363753.1	mangrove clone MKCSB-A2	95-96	2					MBS-16
Crenarchaeota* (95%)	EU427997.1	hydrothermal vent clone 4136-1-83	91		1				GG-8
Crenarchaeota* (86%)	AB213058.1	hydrothermal clone FapmlaA12	84	1					MBS-17
Crenarchaeota* (85%)	EF156623.1	Norris Geyser Basin clone KOZ183	92-95	102	1	1			MBS-13
Desulfurococcales									
*Desulfurcoccus** (96%)	DQ490017.1	boiling spring clone GBS_L3_B06	91-96	20	92	1			Not on tree

APPENDIX A

Supplementary material for Chapter 2, continued. BLAST identification by sample location and archaeal order.

Unclassified Euryarchaeota								
Euryarchaeota	ay940177.1	soil clone CN1	92	1				MBS-9
Euryarchaeota (98%)	AB237749.1	fault clone HDBW-WA16	99			1		LTS-5
Euryarchaeota (98%)	DQ399817.1	anaerobic bioreactor clone PMA5	92-100			6		LTS-4
Euryarchaeota (98%)	AF050616.1	contaminated aquifer clone WCHD3-02	99			2	1	LTS-13
Euryarchaeota (96%)	GQ848379.1	hydrothermal vent clone TVG8AR09	87	1				MBS-7
Unclassified Halobacteria								
Halobacteria	EU869367.1	hypersaline desert clone ARDARCSS3	96	1				MBS-3
Halobacteria	FN391228.1	solar saltern clone SFH1B031	96-97	4				MBS-228
Halobacteria	FN391291.1	solar saltern clone SFH1D081	99	5				MBS-2
Methanobacteriales								
Methanobacterium (97%)	EU519277.1	littoral sediment clone 02-02-502	99			1		LTS-9
Methanothermobacter thermautotrophicus	nr 028241.1	Methanothermobacter thermautotrophicus Marburg	95-99	89	5	3	3	MBS-4, GG-3
Methanomicrobiales								
Unclassified Methanomicrobiales	gu129086.1	Methanomicrobiaceae archaeon 34aM	99				1	C-2
Unclassified Methanomicrobiales (99%)	EU110039.1	marine benthic clone QLS399-A46	95-99				17	C-6

APPENDIX A

Supplementary material for Chapter 2, continued. BLAST identification by sample location and archaeal order.

Unclassified Methanomicrobiales (97%)	EU490290.1	mesotrophic lake clone BIJ2	94-98	1					MBS-1
Methanocalculus	EU721753.1	petroleum reservoir clone D003024H10	96-99					11	C-60
Methanocalculus (98%)	FN548349.1	oil field isolate 4A-22-3f	99					2	C-4
Methanocalculus (95%)	EF592666.1	biofilm reactor clone 2R1A10	96					1	C-21
Methanospirillum	AY454787.1	estuarine sediment clone D_E08	99	3			86	11	UBP-1
Methanospirillum (97%)	DQ841215.1	gas field clone MOB4-1	97-99	5				2	MBS-21
Methanofollis liminatans	nr_028254.1	Methanofollis liminatans GKZPZ	93-99	3	2			22	C-1
Methanosarcinales									
Methanosaeta (100%)	FM162200.1	paper mill clone ARC7_A10	99-100			2			LTS-2
Methanosarcina (99%)	DQ068093.1	subsurface clone MSA22	100			2			LTS-25
Methanosarcina (99%)	EF125517.1	mangrove clone MSASA-B5	98-100			4			LTS-1
Methanosarcina siciliae	U89773.1	Methanosarcina siciliae partial sequence	100			1			LTS-14
Methanosaeta (96%)	AY175381.1	peatland clone LH-02	95-100			1			LTS-16
Thermoplasmatales									
Unclassified Thermoplasmatales	FJ484275.1	Thermoplasmata clone Z17MFA67	95-96				2		UBP-3
Unclassified Thermoplasmatales (98%)	EU118567.1	Anaerobic sludge clone SA_4	99-100				4		UPB-21
TOTAL				**248**	**105**	**34**	**93**	**70**	**550**

APPENDIX B

Supplementary material for Chapter 3. Daily methane produced in micromoles. Average of triplicate cultures, with standard deviations. White indicates less than 25% deviation between cultures, gray indicates 25-100% deviation, dark gray indicates >100% deviation. (A) shows initial experiments, (B) shows pre-exposure experiments.

(A)	1	2	3	4	5	6	8	12	16	20	45	60	72
Control	5.7+/-2	14.3+/-1	8.5+/-6	15.6+/-7	12.3+/-9	25.0+/-15	24.6+/-19	31.6+/-4	28.5+/-4	31.0+/-10	27.7+/-5	14.8+/-11	18.0+/-4
As(III) low	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
As(III) high	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
As(III) Sb	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
As(V) low	7.1+/-1	12.4+/-7	9.4+/-5	6.4+/-3	5.2+/-3	6.5+/-4	6.4+/-5	12.9+/-12	9.6+/-13	4.8+/-6	0+/-0	0+/-0	0+/-0
As(V) high	5.6+/-1	5.4+/-4	2.9+/-2	0+/-0	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
As(V) Sb	6.1+/-2	11.7+/-4	9.3+/-2	6.9+/-0	7.7+/-1	7.3+/-1	7.2+/-2	15.5+/-4	16.8+/-17	10.8+/-13	4.9+/-8	5.2+/-9	5.1+/-9
Sb low	6.6+/-0	18.4+/-2	8.5+/-1	18.3+/-5	18.7+/-6	21.1+/-13	33.4+/-6	29.0+/-4	32.1+/-3	39.8+/-5	24.0+/-1	14.8+/-12	20.0+/-7
Sb high	1.9+/-0	8.2+/-1	7.1+/-2	13.3+/-3	16.4+/-3	26.5+/-5	36.6+/-7	34.9+/-17	32.3+/-17	38.1+/-8	32.2+/-9	20.0+/-13	25.7+/-11
As SC	0+/-0	0+/-0	0.1+/-0	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
Sb SC	0+/-0	0+/-0	0+/-0	0+/-0	0+/-	0+/-0	0+/-0	0+/-0	0.1+/-0	0.1+/-0	0+/-0	0+/-0	0+/-0

APPENDIX B

Supplementary material for Chapter 3, continued. Daily methane produced in micromoles.

(B)	1	2	3	4	7	10	13	14	16	21	25
Low Sb	5.0+/-3	8.2+/-10	10.5+/-13	9.6+/-9	25.0+/-8	21.6+/-7	25.8+/-9	21.5+/-12	14.8+/-8	15.8+/-12	17.2+/-8
Low SB+AsV	9.2+/-3	14.1+/-8	8.6+/-2	8.6+/-0	2.8+/-0	3.8+/-3	19.7+/-21	15.1+/-17	11.3+/-13	0.4+/-1	0.1+/-0
Low Sb+AsIII	0.1+/-0	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
High Sb	1.4+/-1	3.4+/-5	6.7+/-7	9.0+/-5	19.5+/-8	17.5+/-12	20.9+/-17	19.0+/-16	16.8+/-12	10.4+/-6	14.3+/-2
High Sb+AsV	0.3+/-0	0.1+/-0	0.7+/-1	1.0+/-1	1+/-1	0.3+/-1	0.3+/-0	0.2+/-0	0.1+/-0	0+/-0	0+/-0
High SB+AsIII	0.1+/-0	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
Control	3.0+/-1	5.7+/-3	6.5+/-2	10.8+/-3	13.7+/-7	12.7+/-	12.6+/-15	15.4+/-16	6.9+/-12	14.6+/-3	21.1+/-7
SC	0+/-0	0.1+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0

APPENDIX C

Supplementary material for Chapter 4. Taxonomic affiliation of the most abundant OTUs for Bacteria and Archaea,

# seqs	Taxonomic affiliation	Identification	% ID
Bacteria, 211 abundant OTUs			
722	Deinococcus-Thermus	(g) Meiothermus	100
521	Bacteria	(d) Bacteria	100
403	Cyanobacteria	(f) Fischerella	100
383	Betaproteobacteria	(g) Hydrogenophilus	100
322	Chloroflexi	(p) Chloroflexi	98
280	Betaproteobacteria	(f) Tepidimonas	100
212	Deinococcus-Thermus	(f) Thermaceae	100
211	Betaproteobacteria	(c) Betaproteobacteria	100
203	Deinococcus-Thermus	(f) Thermaceae	100
158	Deinococcus-Thermus	(f) Thermaceae	100
137	Betaproteobacteria	(f) Tepidimonas	98
117	Proteobacteria	(p) Proteobacteria	75
114	Betaproteobacteria	(g) Hydrogenophilus	100
112	Betaproteobacteria	(g) Hydrogenophilus	100
109	Bacteroidetes	(g) Crocinitomix	76
105	Deltaproteobacteria	(g) Desulfomicrobium	100
103	Bacteria	(d) Bacteria	100
88	Bacteria	(d) Bacteria	100
80	Verrucomicrobia	(g) Luteolibacter	99
73	Gammaproteobacteria	(g) Kangiella	92
71	Betaproteobacteria	(f) Tepidimonas	98
67	Betaproteobacteria	(g) Hydrogenophilus	100
64	Bacteria	(d) Bacteria	100
64	Bacteria	(d) Bacteria	100
62	Deltaproteobacteria	(g) Desulforhopalus	99
60	Betaproteobacteria	(g) Rubrivivax	77
57	Gammaproteobacteria	(c) Gammaproteobacteria	99
56	Bacteria	(d) Bacteria	100
54	Chloroflexi	(g) Roseiflexus	84
52	Deltaproteobacteria	(g) Desulfovibrio	100
51	Betaproteobacteria	(g) Hydrogenophilus	100
50	Betaproteobacteria	(g) Hydrogenophaga	82

APPENDIX C

Supplementary material for Chapter 4, continued. Taxonomic affiliation of the most abundant OTUs for Bacteria and Archaea,

2b. Archaea, 101 abundant OTUs			
1299	Crenarchaeota	(f) Thermoproteaceae	92
855	Eukaryota	(d) Eukaryota	78
751	Crenarchaeota	(g) Caldivirga	86
645	Archaea	(d) Archaea	86
630	Crenarchaeota	(c) Thermoprotei	95
481	Crenarchaeota	(g) Caldococcus	77
472	Crenarchaeota	(f) Thermoproteaceae	75
418	Crenarchaeota	(c) Thermoprotei	90
350	Euryarchaeota	(g) Methanosaeta	100
346	Korarchaeota	(d) Candidatus Korarchaeum	79
304	Archaea	(d) Archaea	100
304	Crenarchaeota	(c) Thermoprotei	90
228	Euryarchaeota	(g) Methanothermococcus	78
226	Crenarchaeota	(f) Thermoproteaceae	81
225	Crenarchaeota	(c) Thermoprotei	96
212	Crenarchaeota	(g) Caldivirga	83
211	Unclassified	unclassified	
198	Crenarchaeota	(g) Staphylothermus	86
190	Crenarchaeota	(f) Thermoproteaceae	88
179	Archaea	(d) Archaea	99
169	Crenarchaeota	(f) Thermoproteaceae	80
166	Crenarchaeota	(c) Thermoprotei	75
142	Crenarchaeota	(g) Thermoproteus	100
127	Crenarchaeota	(g) Caldivirga	90
125	Crenarchaeota	(g) Caldivirga	83
106	Crenarchaeota	(c) Thermoprotei	90
90	Euryarchaeota	(g) Methanospirillum	100
85	Archaea	(d) Archaea	95
84	Euryarchaeota	(g) Methanothermococcus	93
82	Archaea	(d) Archaea	96
81	Nanoarchaeum	(d) Nanoarchaeum	99
81	Euryarchaeota	(g) Methanothermococcus	82

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